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Pyridostigmine, Permethrin and DEET in Males and Females

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13. ABSTRACT (Maximum 200) The present experiments were conducted to investigate to what extent relatively small doses of PB, PERM and DEET alone, or in different combinations may affect neurobehavioral and immunological outcome in male and female rats. The results of the experiments show that small doses of PB produce neurobehavioral consequences that differ between male and female rats (decrease in locomotor activity, impairment of response acquisition). They also show that gonadal hormones affect PB kinetics as higher PB levels were observed in female rats than in male rats. PB levels were higher in pro-estrus females than in met-estrus female rats and intact male rats. PERM and DEET administration alone did not greatly affect locomotor activity (and response acquisition in the case of PERM), but some synergistic effects were observed when they were administered together with PB. In this context it is important to note that, PB administration changed PERM serum levels as they were much higher when PERM was coadministered with PB. PERM levels were higher in female rats than in male rats. It should be noted that these effects were observed in rats that were free of stress other than that inflicted by participation in the research protocol.			
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FOREWORD

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INTRODUCTION

Some of the 650,000 male and 50,000 female US soldiers who served during the Gulf War were exposed to prophylactic doses of the cholinesterase inhibitor pyridostigmine bromide (PB) possibly in combination with pesticides such as permethrin (PERM) and insect repellents such as N,N,-Diethyl-M-Toluamide (DEET). Very little information is available concerning the neurobehavioral and immunological toxicity of these compounds, but it has been hypothesized that this exposure may have contributed toward symptoms associated with the 'Gulf War Syndrome'.

The main hypothesis of this multidisciplinary research effort is that the administration of PB, PERM and DEET as single agents, or in combination, results in neurobehavioral toxicity and an altered immune response which may differ between male and female subjects. To evaluate this hypothesis, the behavior of adult male and female rats will be studied in experiments designed to measure various aspects of CNS functioning in the presence of sub-toxic doses of PB, PERM, and DEET. The neurobehavioral analyses are complemented by an assessment of the immune response in rats and lymphocytes from healthy human volunteers.

BACKGROUND

It has been suggested that exposure to PB, PERM and DEET may have played a role in the development of the syndrome which appears to have afflicted some of the military personnel who served during the Gulf War (Almog, et al., 1991).

PB is a quaternary ammonium compound that is classified as an anticholinesterase agent. It inhibits the hydrolysis of acetylcholine (ACh) by competitive reversible binding to acetylcholinesterase. PB decreases nerve gas toxicity by occupying acetylcholinesterase binding sites. Although PB and nerve gas share the same mechanism of action, PB is much less toxic due to the reversible binding and the short duration of action. During the Gulf War, PB was taken prophylactically when there was a high risk of exposure to nerve gas.

The pyrethrins, of which PERM is a synthetic example, are considered to exhibit low acute toxicity since they are rapidly hydrolyzed in the gastrointestinal tract following oral ingestion and by liver esterases in the blood (Metcalf and McKelvey, 1974). Hydrolysis of PERM results in the production of pyrethrin, and pyrethroid alcoholic, phenolic or carboxylic acid metabolites which are excreted as the glycine, sulfate, glucuronide or glucoside conjugates (Czasida, et al., 1983; Miyamoto, 1976). In sufficient concentrations however, pyrethrins have been shown to be neurotoxic, with effects including hypersensitivity, tremors and seizures (Dorman and Beasley, 1991). During the Gulf War this compound was used to impregnate some battle-dress uniforms in the field.

DEET is the most commonly used insect repellent in the world (Veltri, et al. 1994). It has also been proposed as a pharmaceutical excipient to improve dermal and transdermal delivery of drugs (Windheuser, et al., 1982). The exact mechanism of DEET toxicity is unknown, but pathological findings indicate that it is a demyelinating agent, which causes spongiform

myelinopathy (Verschoyle, et al, 1992). DEET was available during the Gulf War, but used infrequently.

PB, PERM and DEET have all been used as individual agents with an apparent low rate of adverse events. Some recent evidence suggests, however, that the neurotoxicological effects of combinations of these agents may exceed their individual effects. McCain (1995) has reported that large oral doses of PB, PERM and DEET kill male laboratory rats, either when the compounds are administered simultaneously, or when PB is administered together with PERM or DEET. The effects were greater than additive, except when DEET and PERM were administered concurrently. It is not known whether similar results would have been obtained in female subjects, but there are reasons to assume that the neurobehavioral effects of these different compounds may be gender-dependent (Barbarino, et al., 1991; O'Keane and Dinan, 1992).

RESEARCH ACCOMPLISHMENTS

Changes in locomotor activity are an important source of information to the behavioral toxicologist as they provide a first indication of a compound's behavioral effects when administered in subtoxic doses. A number of experiments were conducted to study the effects of PB alone, or in combination with PERM and/or DEET, on locomotor activity in adult male and female rats. In different experiments, PB, PERM and DEET were administered acutely or repeatedly over a limited period of time.

In one of these experiments (Hoy, van Haaren, Tebbett and Karlix, 1997; Hoy, Cody, Karlix, Schmidt, Tebbett, Toffolo and Wielbo, 1999), we administered different doses of PB (0, 3, 10 and 30 mg/kg) to male and female Sprague-Dawley rats, 30 min prior to the analysis of their locomotor activity. Different groups of female rats were tested during the pro-estrus and met-estrus parts of their cycles. PB was administered by gavage and locomotor activity was recorded for two hours. The locomotor responses habituated over the course of the two hours of observation in all groups of rats. We observed that locomotor activity decreased significantly and that the rats spend significantly more time against the walls of the open-field (index of anxiety) as the dose of PB was increased. The behavior of female rats was more affected than that of males. PB serum levels were higher in female rats than in male rats. Following the highest dose of PB, PB serum levels were much higher in pro-estrus females than in males and met-estrus females. This experiment was completed in year 1 of the grant in accordance with the initial Statement of Work (Experiment 1-1). Full details may be obtained from the published paper enclosed as Appendix 1 (Hoy, J.B.; Cody, B.A.; Karlix, J.L.; Schmidt, C.J.; Tebbett, I.R.; Toffollo, S.; van Haaren, F.; Wielbo, D. Pyridostigmine bromide alters locomotion and thigmotaxis of rats: gender effects, *Pharmacology, Biochemistry and Behavior* 63(3): 401-406, 1999).

We also studied the effects of PB, PERM and DEET, alone or in combination, on locomotor activity in male and female rats. All drugs or drug combinations were administered only once (acutely). Different groups of female rats were tested during the met-estrus and pro-estrus parts of their cycles. Dose-effects curves were determined for PERM (0, 15, 30 or 60 mg/kg, IP) and

DEET (0, 50, 200, 500 mg/kg, by gavage), but dose-related effects on locomotor activity were not observed when these chemicals were administered alone. The results of this experiment further showed that combinations of PB and PERM and combinations of DEET and PERM significantly decreased locomotor activity in male rats compared to administration of double the dose of the individual compounds. Furthermore, coadministration of DEET and PERM also synergistically affected anxiety in male rats. Interestingly, synergistic interactions were not observed in the female rats, nor were there any differences between groups of female rats as a function of estrus cycle. Serum samples obtained following a second administration of all three chemicals were also analyzed. PB and DEET serum levels appeared higher in metestrus and proestrus females than in males when PB and DEET were administered at 10 mg/kg and 200 mg/kg, respectively. There were no differences between males and females in PERM serum levels following administration of 30 mg/kg. However, when PERM was administered at 15 mg/kg in combination with a small dose of PB (5 mg/kg) much more PERM was detected in serum samples. This experiment was completed in years 2 and 3 of the grant in accordance with the initial Statement of Work (Experiment 1-2, 1-3, 1-4, 1-5, 1-6). Full details may be obtained from the published paper enclosed as Appendix 2 (Hoy, J.B.; Cornell, J.A.; Karlix, J.L.; Schmidt, C.J.; Tebbett, I.R.; van Haaren, F. Interactions of pyridostigmine bromide, DEET and Permethrin alter locomotor behavior of rats. Veterinary and Human Toxicology, 42(2): 65-71, 2000).

In another experiment, PB, PERM and DEET were repeatedly administered alone, or in all possible combinations, to male and female Sprague-Dawley rats. Repeated administration occurred over the course of seven consecutive days and the effects were measured 24 hours following the final drug administration (Appendix 3). Drug doses were chosen on the basis of the behavioral effects observed in previous experiments. In this experiment, PB was administered at 7.5 mg/kg (alone), 3.75 mg/kg (in combination with PERM or DEET) or at 2.5 mg/kg (in combination with PERM and DEET). PERM was administered at 60 mg/kg (alone), at 30 mg/kg (when combined with PB or DEET) and at 20 mg/kg (when combined with PB and DEET). DEET was administered at 200 mg/kg (alone), or at 100 mg/kg (when administered with PB or PERM), and at 67 mg/kg (when administered with PB and PERM). PB, PERM and DEET, when administered alone for seven consecutive days did not produce behavioral effects different from those observed in control subjects. Twenty-four hours after the final drug administration, locomotor activity in males and females given combinations of PB and DEET was lower than that observed in control subjects. Locomotor activity in males given DEET and PERM was higher than that observed in control subjects and females that had received combinations of PB and PERM for seven consecutive days spent significantly more time than control subjects in the center of the arena. Other differences between experimental groups and control groups were not observed. This experiment was completed in years 2 and 3 of the grant in accordance with the initial Statement of Work (Experiment 1-7 and 1-8). Full details may be obtained from the published paper enclosed as Appendix 3 (Hoy, J.B.; Cornell, J.A.; Karlix, J.L.; Tebbett, I.R.; van Haaren, F. Repeated coadministrations of pyridostigmine bromide, DEET and permethrin alter locomotor behavior of rats. Veterinary and Human Toxicology, 42(2): 72-76, 2000).

In other experiments we were to investigate the effects of PB, PERM and DEET on learning and performance in male and female rats. Three experiments were originally included in the Statement of Work. Experiment 2 (year 1) involved an analysis of the effects of PB, Perm and DEET alone, or in combination on multiple fixed-interval, fixed-ratio performance in intact male and female rats, Experiment 3 (year 2) was designed to investigate the effects of PB, Perm and DEET, alone or in combination on progressive ratio performance in intact male and female rats, while Experiment 4 (year 3) was designed to establish the effects of PB, Perm and DEET alone, or in combination on delayed non-matching to position performance in intact male and female rats. During years 1 and 2 it became evident that we had grossly underestimated the amount of time that it would take to conduct Experiments 2, 3 and 4. These studies were all designed to assess the effects of PB, Perm and DEET on well-established behavior in individual male and female rats. Drug effects could only be studied if baseline rates of behavior were stable. It turned out that it was rather difficult to maintain stable baseline rates of behavior in Experiment 2. In view of these observations, which made it very likely that we would not be able to complete Experiments 2, 3 and 4 within the time frame of the grant, we decided to add experiments that would not take a long time to conduct, and that would also provide important information with respect to the effects of PB and Perm on learning measures. DEET was not included at this time because it appeared from our initial observations in studies of locomotor activity that the behavioral effects of DEET would be negligible. Experiment A (year 2) was added to study the effects of acute and repeated PB administration on response acquisition with immediate and delayed reinforcement. Experiment B (year 2 and 3) was added to study the effects of acute and repeated PB administration on response acquisition in male and female rats. Experiment C (year 2 and 3) was added to study the effect of PB and Perm, alone or in combination, on response acquisition in male and female rats.

In Experiment A (van Haaren, de Jongh, Hoy, Karlix, Schmidt, Tebbett and Wielbo, 1999) different groups of male Sprague-Dawley rats either received one acute administration of PB at 10 mg/kg or repeated administration of PB (seven days at 1.5 mg/kg). Control groups were treated with distilled water. All subjects were then exposed to an experimental procedure in which they had to learn to press a lever to obtain a food pellet. Food pellet delivery was either immediate or delayed by 16-s to manipulate the difficulty of the task. This was a successful manipulation as all subjects learned much better when pellets were delivered immediately than when they were delivered after 16 s. Acute and repeated PB administration produced the same behavioral effects. PB administration delayed the onset of responding in some, but not all, of the subjects in the treated groups independent of the delay condition to which they were exposed. Many more responses were observed on an inoperative lever during the 16-s delay conditions than during the 0-s delay conditions, especially during the 16-s delay condition in which subjects had received acute administration of the PB vehicle. This experiment was completed in year 2 of the grant as an addition to the initial Statement of Work (Experiment A). Full details may be obtained from the published paper enclosed as Appendix 4 (van Haaren, F.; de Jongh, R.; Hoy, J.B.; Karlix, J.L.; Schmidt, J.; Tebbett, I.R.; Wielbo, The effects of acute and repeated pyridostigmine bromide administration on response acquisition with immediate and delayed reinforcement. Pharmacology Biochemistry and Behavior, 62(2): 389-394, 1999).

In Experiment B, male and female Sprague-Dawley rats were treated with different doses of PB (3 or 10 mg/kg) either once, or for fourteen consecutive days (van Haaren, Turner, Cody, Hoy, Karlix, Schmidt, Tebbett and Wielbo, manuscript in revision). Response acquisition was then assessed 30 min following the final PB administration. Acute or repeated administration of 10 mg/kg PB inhibited the acquisition of a novel response in male and female rats alike.

Differences between vehicle administration and the 3 mg/kg dose of PB were not observed, most likely because repeated vehicle administration already impaired response acquisition in male rats, but not in female rats. We suggested that the latter effect might have been caused by the repeated stress associated with PB administration via gavage. Following PB administration, female rats were more likely than male rats to contact a lever on which no consequences were programmed (errors). Full details of methodology and results appear below, as these data have not yet been published.

METHODS

Subjects. Experimentally naive, male and female Sprague-Dawley rats were obtained from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN) when they weighed between 250-275 g. They were housed in-groups of three under a reversed, 12-h, light-dark cycle (lights on 6:00 p.m.) in a temperature and humidity controlled environment. The rats were handled daily for two weeks before the beginning of the experiment. Standard rodent chow was available in the home cages during the first week. Starting with the second week, rodent chow was limited to approximately 12 g per female rats and 16 g per male rat. Water was continuously available in the home cages

Apparatus. The experiments were conducted in six rodent operant conditioning chambers (Coulbourn Instruments, Allentown, PA). The chambers were 25 cm wide, 30 cm long and 29 cm high. The sidewalls were made of Plexiglas; the intelligence panel and the back wall consisted of modular stainless steel panels. The floor consisted of 16 rods, spaced 1.75 cm apart. A pellet tray was located 1.7 cm above the floor in the middle of the intelligence panel and a houselight was approximately 3 cm from the ceiling of the chamber. The pellet tray could be illuminated during pellet presentation (Noyes, 45-mg rodent purified formula). There were two retractable levers, one to the right and one to the left of the pellet tray. They were spaced 12.5-cm apart and located 6.3 cm above the floor. When extended, the levers protruded 1.8 cm from the intelligence panel. Each chamber was enclosed in a sound-attenuating and ventilated cubicle. Experimental events were controlled and data were collected using an IBM compatible computer (GatorByte, Gainesville, FL) with L2T2 software and LabLinc interfacing obtained from Coulbourn Instruments (Allentown, PA).

Procedure: The male and female rats were divided into groups of six rats each that were exposed to one of six different experimental conditions. The drugs were administered either acutely or repeatedly and the subjects received either PB (3 or 10 mg/kg) or distilled water (vehicle). When the drugs were administered acutely, the rats were first trained to retrieve food pellets from the tray in the operant chamber (magazine training). Then, for three consecutive days, they received

distilled water by gavage. They were tested 30 min following PB or vehicle administration on day four. When the drugs were administered repeatedly, the rats were also first trained to eat from the magazine. Then, for 14 consecutive days, they received either PB (3 or 10 mg/kg) or distilled water by gavage and they were tested 30 min after drug or vehicle administration on day 14.

Magazine training. During magazine training, the rats were first placed in the darkened operant chamber from which both levers had been retracted. After five minutes, the houselight was illuminated and food pellets were delivered into the pellet tray on a variable time (VT) 60-s schedule. Both levers remained retracted during the magazine training session that was terminated after 60 pellets had been delivered.

Response acquisition. The response acquisition session also began with a five-minute dark period during which the levers were retracted from the chamber. Then, the houselight was illuminated and both levers were extended into the operant chamber. Pressing the left (operative) lever immediately resulted in pellet presentation. Pressing the right (inoperative) lever had no scheduled consequences. The experimental session was terminated after eight hours.

Drugs. Pyridostigmine bromide (PB, Sigma Chemical Co, St. Louis, MO) was dissolved in distilled water. PB (3 or 10 mg/kg) and distilled water were administered by gavage, in a volume of 5 ml/kg, 30 min prior to the beginning of the experimental session (acute administration) or for 14 days, each day at approximately 30 min prior to the scheduled starting time of the experimental session on day 14 (repeated administration).

Serum preparation. Trunk blood was collected from male and female rats ($n=3$ per group) that had not participated in the behavioral experiments but that had been treated identically in terms of housing conditions and drug administration. To collect blood, the rat was placed in a jar containing a paper towel saturated with Metofane (Methoxyflurane), 30 min after PB or vehicle administration. The anesthetized animal was quickly decapitated after one min. Blood was collected in a 15-ml polystyrene culture test tube and allowed to coagulate on ice for two hours. It was then centrifuged for 15-20 minutes at approximately 3000 revolutions per minute. The serum was then drawn off the solid cell matter with a clean glass Pasteur pipette and placed in a 1.5 ml polystyrene microcentrifuge tube. It was immediately placed in a freezer (at -70 degrees Fahrenheit) where it was stored until further analysis.

Serum analyses

Pyridostigmine bromide. Serum samples and serum PB spiked standards (1.0 ml) were vortexed with 2.0 ml of 0.5M potassium phosphate buffer (pH 10.5). The mixture was applied to a C18 Prep extraction column (Fisher Scientific p-453) which had been conditioned prior to use with 5.0 ml of methanol followed by 5.0 ml of distilled water. Following sample application the column was washed with 5.0 ml of 0.05 ml potassium phosphate buffer (pH 10.5) and 5.0 ml methanol. PB was eluted with 3.0 ml of 1-% acetic acid methanol, evaporated to dryness under nitrogen and reconstituted in 200 micrometers of mobile phase-A (MP-A). A 50 microliter aliquot was then applied to an Ultrasphere Octyl column, 5 microns, 4.6 mm x 25 cm (Beckman Instruments, Inc., Fullerton, CA). The high performance liquid chromatographic system (HPLC)

consisted of a Hewlett-Packard (HP) 1100 series quaternary pump, a HP 1100 series Thermostatted Column Compartment, a HP 1100 series Autosampler, a HP 1100 series vacuum degasser, a HP 1100 series variable wavelength detector operated at 208 nm and a HP Chemstation for LC Systems software. Mobile phase consisted of low pressure mixing of two solvent systems (MP-A and MP-b) at 50% (volume) for each by the 1100 series Quaternary pump. MP-A consisted of acetonitrile/water (30:70), 0.1% sodium Lauryl sulfate (wt/vol.), 0.1% H₃PO₄ (vol./vol.) and 0.0025M tetramethylammonium chloride. MP-b consisted of acetonitrile/water (30:70), 0.4% sodium Lauryl sulfate (wt/vol.), 0.1 H₃PO₄ (vol./vol.). Quantitative analysis was achieved by comparison of peak areas with extracted serum standards over the range of 0.0 - 350.4 nanograms per ml of serum. Flow rate was 1.0 ml per minute. Column temperature was maintained at 25 degrees Centigrade by the HP 1100 series Thermostatted Column Compartment.

Serum cholinesterase. Prepared test kits (Sigma, St. Louis MO, 420-MC) were used to measure cholinesterase activity. This assay is based on the method of Rappaport, et al. [14] and depends on the quantitative formation of acetic acid from acetylcholine in the presence of an acid-based indicator, m-nitrophenol. All assays were done in triplicate.

Brain cholinesterase. Half a brain (approximately 0.9 g) was placed in a 15-mL conical polypropylene tube with 5 mL of Dulbecco's Phosphate buffered salt solution. The tissue was homogenized in a Tissue Tearor (model 985-370) for about 2 min. Tubes were then capped and centrifuged at 4000 rpm for 20 minutes at 4 degrees C. The supernatant was then assayed as described above.

Statistical analyses. The total number of responses on the active lever were evaluated with Analysis of Variance (ANOVA) involving the factors, Sex (male or female), Treatment (acute or repeated), Drug (0, 3 or 10 mg/kg PB) and Time (repeated, at each full hour of the experimental session). Responses on the inactive lever were also evaluated with ANOVA. Serum and brain cholinesterase levels were evaluated with ANOVA involving the factors, Sex, Treatment and Drug.

RESULTS

Figure 1 shows the cumulative number of reinforced responses for individual male and female rats following acute exposure to either distilled water, 3 mg/kg PB or 10 mg/kg PB. Open circles represent the number of responses on the inactive lever averaged over all subjects in the group.

Insert Figure 1 about here

Figure 2 shows the cumulative number of reinforced responses for individual male and female subjects following repeated exposure (14 days) to distilled water, 3 mg/kg PB or 10 mg/kg PB. Open circles represent the group-averaged number of responses on the inactive lever.

Insert Figure 2 about here

Analysis of variance showed that the number of responses on the active lever increased as a function of time in the experimental session ($F(7,427) = 27.00, p < 0.0001$). The cumulative number of responses on the active lever did not vary as a function of treatment (acute or repeated) or sex (male or female), but decreased as a function of PB dose ($F(2,59) = 4.32, p < 0.0178$). Post-hoc analyses revealed no differences between untreated subjects and those who had received 3 mg/kg PB, but a comparison between these two groups of subjects and those who had received 10 mg/kg PB revealed that response acquisition was decreased in the latter group of subjects.

Female rats were more likely than male rats to respond on the inactive lever ($F(1,60) = 8.57, p < 0.0048$). More errors were made over time ($F(7,426) = 18.10, p < 0.0001$) and they increased as a function of drug administration ($F(2,60) = 0.043$). Significant interactions between sex and time of session ($F(7,426) = 2.26, p < 0.0288$) and drug and time of session ($F(14,426) = 1.74, p < 0.0461$) showed that females made more errors than males over time and that more errors were made following the administration of 3 mg/kg PB than following 10 mg/kg PB (Figures 1 and 2).

PB serum levels were determined in rats that had not participated in the behavioral experiments, but that, otherwise, had been treated identical to those who had. Thirty minutes following the first or fourteenth administration of 3 mg/kg PB, its serum levels could not be reliably determined in our assay. Following acute and repeated administration of 10 mg/kg PB, PB levels (nanograms/milliliter) averaged 94.9 (range 40.6-144.6) and 139.91 (89.6-178.9) in male and female rats (acute) and 72.2 (30.9-110.33) and 87.4 (53.3-114.5) in male and female rats (repeated). PB serum levels were not different between male and female rats due, most likely, to the large between-subject variability in the observations.

Table 1 shows serum cholinesterase levels (Rappaport units) in male and female rats following PB administration. Baseline serum cholinesterase levels were higher in female rats than in male rats ($F(1,24) = 6.71, p < 0.016$). PB administration dose-dependently decreased serum cholinesterase levels ($F(2,24) = 6.91, p < 0.0043$, independent of treatment condition ($F(1,24) = 0.79, n.s.$). Post-hoc tests showed that administration of 3 mg/kg and 10 mg/kg PB significantly reduced serum cholinesterase levels as compared to controls. The difference between the administration of 3 mg/kg PB and 10 mg/kg PB was not significant. There were no differences in baseline *brain* cholinesterase levels between males and females, nor did any of the treatment conditions significantly affect brain cholinesterase levels.

Insert Table 1 about here

In experiment C, response acquisition in male and female rats was analyzed following PB and Perm administration alone, or in combination (van Haaren, Cody, Hoy, Karlix, Schmidt, Tebbett and Wielbo, 2000). Male and female Sprague-Dawley rats were treated with PB (1.5 mg/kg) or distilled water for seven days. They then also received an IP injection of PERM (0, 15 or 60 mg/kg) immediately before the learning session. Serum PERM levels increased as a function of its dose and were higher in rats treated with PB. Sex differences were also observed as PERM levels were higher in female rats than in male rats. PB administration delayed learning in male

and female rats and females made more errors than males. Although PERM levels were higher in subjects treated with PB, there were no differences in the behavioral effects of PERM. Full details may be obtained from the paper which is in press and enclosed as Appendix 5 (van Haaren, F.; Cody, B.; Hoy, J.B., Karlix, J.L., Schmidt, C.J., Tebbett, I.R.; Wielbo, D. The effects of pyridostigmine bromide and permethrin alone, or in combination, on response acquisition in male and female rats. Pharmacology Biochemistry and Behavior, in press, 2000).

During years 2 and 3 we also initiated experiments to evaluate the effects of PB, Perm and DEET alone, or in combination on multiple fixed-interval, fixed-ratio performance in intact male and female rats (Statement of Work Experiment 2) and to investigate the effects of PB, Perm and DEET, alone or in combination on progressive ratio performance in intact male and female rats (Statement of Work, Experiment 3). At this time, data collection and data analysis is not complete.

The exact etiology and pathophysiology of the Gulf War syndrome are poorly understood. Very little information is available on the immunotoxicological effects of these compounds. Many of the signs and symptoms of the Gulf War Syndrome are similar to those of chronic fatigue syndrome including joint pain, fatigue and depression. Like the Gulf War Syndrome [GWS], the etiology and pathophysiology of chronic fatigue syndrome is unclear. One of the primary causes of chronic fatigue syndrome is hypothesized to be focused upon immune dysfunction (Downey, 1992; Holmwood and Shannon, 1992; Murdoch, 1992; Blondel-Hill and Shafran, 1993). Because the GWS signs and symptoms are so similar to those reported in chronic fatigue syndrome, studies were undertaken to evaluate the immunotoxicological effects of PB, PERM and DEET (Karlix, Freiburger, Hoy, Tebbett, Wielbo, Schmidt, Myers, van Haaren, manuscript submitted for publication). Statement of Work, Immunology Experiment 1.

This study was undertaken to investigate whether certain chemicals that soldiers were exposed to during the Gulf War possess any immunomodulatory effects. Human lymphocytes were isolated and exposed to varying concentrations of PERM, PB and DEET. The human lymphocytes were stimulated via mitogens PMA [phorbol-12-myristate 13-acetate], PHA [phytohemagglutinin], and MLR [mixed lymphocyte response] and immune response was measured either as immunostimulation or immunosuppression. All three agents demonstrated a dose-dependent response. Perm and DEET showed the greatest immunomodulatory activity with statistical differences against controls in the PMA, PHA and MLR as measured by cpm. PERM IC₅₀'s were 4.8 ug/ml PMA, 7.5 PHA and 46 ug/ml MLR. DEET was not as potent as PERM with IC₅₀'s of 100 ug/ml PMA, 95 ug/ml PHA and 50 ug/ml MLR. In contrast to the other agents, PB did not reach an IC₅₀ but showed immunostimulation at low concentrations. All three agents demonstrated immunomodulatory effects that must be considered when addressing the pathophysiology of the Gulf War Syndrome.

METHODS

Isolation of peripheral blood mononuclear cells (PBMCs): PBMCs were isolated from healthy volunteers using a previously described protocol (Karlix, J.L. Cocaine suppresses fetal immune system. (1998), Pediatric Research, 44(1), 43-46.) All chemicals were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise stated. The blood draw procedure was approved through the Shands Hospital Investigational Review Board (protocol #512-95). Briefly, the peripheral blood was collected and the lymphocytes were isolated via a ficol hypaque density gradient. The cell volume was adjusted to achieve a concentration of 1×10^6 cells/ml for phytohemagglutinin (PHA) and phorbol-12-myristate 13-acetate (PMA) and 2×10^6 cells/ml mixed lymphocyte response (MLR) with 5% HSA.

Cell proliferation assay In the mitogen assays, PHA was added at a concentration of 5 ug/ml, while PMA was added at a concentration of 50 ng/ml. One hundred microliters of the cell solutions were placed into their corresponding wells of a 96 well microtiter plate with an equal volume of chemical diluted in RPMI with 5% HAS. Blood from two volunteers was required for a MLR assay. The cells from one volunteer were irradiated with 56Cs gamma emitter for 2500 rads of total exposure. The irradiated cells were used as the stimulator cells. Both irradiated and non-irradiated cells (responder cells) were transferred to a 96 well culture plate (50 ul each). The cells were combined with one hundred microliters of chemical or control. The controls for all assays included stimulated and unstimulated cells with RPMI containing 5% HAS, stimulated cells with methanol control, and RPMI with 5% HAS. The methanol control was only assayed when the agent being studied was insoluble in aqueous solution and had to be first diluted with methanol (DEET and Perm). The concentration of the methanol control was based on the highest point of the standard curve in a volume to volume ratio. The microtiter plate was covered and incubated at 37 C with 5% CO₂. Following 48 hours incubation period for PHA and PMA, and 120 hours for MLR, each well was radiolabelled with 1 uCi of [³H]-thymidine (Nen Life Science products, Inc, Boston, MA) and incubated for an additional 24 hours at 37 C. The radiolabelled cells were then harvested on a Skatron filtermat paper using an automated cell harvester (Skatron, Inc, Sterling, VA). The filters were transferred to vials with National Diagnostics Ecoscint-O scintillation cocktail (Atlanta, GA). Each sample was then read on a Beckman LS 6500 scintillation counter to assess the presence of [³H]-thymidine incorporation by the cells and reported in counts per minute (Fullerton, CA). In each tissue culture plate, all samples were performed in triplicate. All experiments were repeated three times. Percent inhibition was calculated according to the following equation:

$$\% I = ((\text{stimulated cells exposed to chemical-stimulated cells}) / \text{stimulated cells}) * 100$$

Statistical analysis Data included all triplicate sample data in each of the three repeated experiments and is reported in counts per minute +/- one standard deviation. The Dunnett's t test for comparison to control was used to analyze the data. A percent effect was calculated from the raw data, therefore no statistical analysis was performed on these numbers.

RESULTS

All three agents singularly affected the immunoassays in a dose-dependent manner. Mitogen counts per minute data are shown in Tables I1-I3.

Insert Tables I1-I3 about here

Percent inhibition: The above data were calculated to determine the percent effects of a chemical to inhibit or to stimulate the immune response. Because the raw data were used in the calculation to determine the percent effect, no statistical analysis was performed on the calculated numbers. Since the primary effects in the concentration range tested were inhibition, the calculations were presented in graph form as percent inhibition. The percent inhibitions are presented in low (data not shown in tabular form) and high concentration ranges for each chemical (Figures I1-I6).

Insert Figures I1-I6 about here

Permethrin demonstrated minimal immunostimulatory effects from 0-1 ug/ml with the maximum immunostimulation of 5% shown in the PMA stimulated cells. The inhibitory concentration by which 50% of the response was inhibited (IC50) was 4.8 ug/ml for PMA stimulated cells, 7.5 ug/ml for PHA stimulated cells, and 46 ug/ml for the MLR (Figures I1 and I2). Like permethrin, DEET showed moderate immunostimulatory effects from 0-2 ug/ml with the maximum immunostimulation of 8% shown in the MLR. The retrospective IC50s were 100 ug/ml for PMA stimulated cells, 95 ug/ml for PHA stimulated cells, and 50 ug/ml for the MLR (Figures I3 and I4). Pyridostigmine bromide demonstrated the greatest immunostimulatory effects as compared to the other agents. Immunostimulatory effects from 0-10 ug/ml with the maximum immunoistimulation of 15% shown in the MLR. In contrast to the other agents, pyridostigmine bromide did not reach an IC50 for any of the immunoassays (Figures I5 and I6).

During years 2 and 3 the materials to conduct Immunology Experiments 2 and 3 were obtained from rodent subjects (serum and spleens) that participated in locomotor experiments 1-4, 1-5, 1-6, 1-7, 1-8 and from human subjects (lymphocytes). At this time, Dr. Karlx has not yet completed the data analysis and inclusion in this report is not warranted.

REPORTABLE OUTCOMES

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van Haaren F, Turner SM, Cody BA, Hoy JB, Karlix JR, Schmidt CJ, Tebbett IR, Wielbo D. The effects of acute and repeated pyridostigmine bromide administration on response acquisition in male and female Sprague-Dawley rats, in preparation.

CONCLUSIONS

The present experiments were conducted to investigate to what extent relatively small doses of PB, PERM and DEET alone, or in different combinations affect neurobehavioral and immunological outcome in male and female rats. The results of the experiments show that small doses of PB produce neurobehavioral consequences that differ between male and female rats (decrease in locomotor activity, impairment of response acquisition). They also show that gonadal hormones affect PB kinetics as higher PB levels were observed in female rats than in male rats. PB levels were higher in pro-estrus females than in met-estrus female rats and intact male rats. PERM and DEET administration alone did not greatly affect locomotor activity (and response acquisition in the case of PERM), but some synergistic effects were observed when they were administered together with PB. In this context it is important to note that PB administration changed PERM serum levels, as they were much higher when PERM was coadministered with PB and they were higher in female rats than in male rats. It should be noted that these effects were observed in rats that were free of stress other than that inflicted by participation in the research protocol.

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Pyridostigmine Bromide Alters Locomotion and Thigmotaxis of Rats: Gender Effects

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HOY, J. B., B. A. CODY, J. L. KARLIX, C. J. SCHMIDT, I. R. TEBBETT, S. TOFFOLLO, F. VAN HAAREN AND D. WIELBO. *Pyridostigmine bromide alters locomotion and thigmotaxis of rats: Gender effects.* PHARMACOL BIOCHEM BEHAV 63(3) 401–406, 1999.—Male rats and female rats in the proestrous and metestrous stages of estrus were tested to determine the effects of pyridostigmine bromide on locomotion rate and thigmotactic response using doses of 3.0, 10.0, and 30.0 mg/kg. Thirty minutes after administration of the pyridostigmine bromide the rats were videorecorded for 2 h in a 1 m² open-field arena. The rats' activities were analyzed for the drug's effect on speed throughout the 2 h and during six 20-min segments. Also, the times that the rats were observed moving through the central 50% of the arena were determined. Locomotion rates decreased significantly, and thigmotaxes increased significantly in all groups of rats as a dose response to pyridostigmine bromide. Habituation occurred over 2 h for both responses, primarily during the first 40 min. Female rats were more affected than males, but metestrous and proestrous females did not differ significantly in their responses. At the 30 mg/kg the effect was persistent throughout the test period. Proestrous females dosed at 30 mg/kg had much higher pyridostigmine bromide serum levels than metestrous females and males. © 1999 Elsevier Science Inc.

Pyridostigmine bromide Locomotion Open field Thigmotaxis Gender effect

PYRIDOSTIGMINE bromide (PB) is an acetylcholinesterase inhibitor that has been used as a treatment for myasthenia gravis for many years (8). A recent study has shown a synergistic effect between DEET and both PB and permethrin when administered to cockroaches (15). Coexposure to PB, *N,N*-diethyl-*m*-toluamide (DEET), and permethrin has also been shown to have synergistic behavioral effects in chickens (1). A synergistic effect (LD₅₀) of coexposure to PB, DEET, and permethrin using male rats has been reported (12). In this case, oral administration of PB in propylene glycol resulted in estimation of LD₅₀ of 61.6 mg/kg. Combinations of the three drugs at dosages calculated to cause mortality of 48% of the animals caused mortalities of 80 to 90%.

Sublethal effects of neurotoxic compounds may be seen in various measures of locomotor activity (4,9,14). Neurobehavioral screening of pesticide effects on mammals has been reported (13). Low doses of PB (3–12 mg/kg) decreased response frequency during operant tests (17). Gender and estrous cycle were identified as factors in reduced open-field activity produced by interleukin-1b (2). Similarly, gender dif-

ferences in susceptibility of cockroaches to toxicants has been reported (10). Open-field locomotor activity in rats, using automated data acquisition, can show chemically induced changes in speed and thigmotactic responses (3,4,9,16). Significant changes in the open-field behavior of rats dosed with PB at 5.5% of LD₅₀ have been reported (19). In this case, intraperitoneal administration of PB resulted in estimation of LD₅₀ at 2699 mg/kg.

The purpose of this study was to determine the effects of PB on locomotor and thigmotactic activity of male rats and female rats in proestrous and metestrous. Furthermore, we sought baseline information for future study of the synergistic effects of PB, DEET, and permethrin on locomotion.

METHOD

Subjects

Sprague-Dawley rats (250 g) were obtained from Harlan-Sprague-Dawley (Indianapolis, IN), and housed same sex, two per cage, under a reversed light cycle of 12 D:12 L (lights

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on 1800 h), and fed rat chow ad lib. The rats were identified by ear-punch code. Each rat was handled about 30 s 5 days/week for at least 7 weeks prior to testing. Treatments were assigned to individuals at random within groups and time of test. Tests were done between 900 and 1700 h. Male, and proestrous, and metestrous female rats were tested two at a time in individual arenas. Male rats were treated first, and whenever possible metestrous females were tested second and proestrous females last. Alternatively, only females of one type were tested if both types were not available on a given day. All dosing and handling of test subjects were done by the same technician.

Estrous Stage Determination

Female subjects were examined 1 to 3 h before testing to determine their estrous cycle status. The criteria for assignment to proestrous or metestrous categories was based on microscopic examination of epithelial cells found in the vaginal fluid of the rats.

Drug and Dosage

PB obtained from Sigma (St. Louis, MO) was orally administered by gavage tube in distilled water at low, medium, and high doses, 3.0, 10.0, and 30.0 mg/kg, respectively, in a volume of 5 ml/kg. Control animals were dosed with matching volumes of distilled water. Test subjects were held 30 min prior to introduction to the test arenas, then placed in the center of the arena about 30 s prior to recording of their activity.

Arenas

The tests for locomotor activity were done in two black ABS plastic arenas that were 100 × 100 × 30-cm high. Each arena was surrounded by a black curtain. The arenas were on opposite sides of a rack that supported lights, video cameras, and video cassette recorders. Indirect low intensity light was provided by three 60-watt red bulbs approximately 2.2 m above each arena, and located so that the center of each arena received about 2 lx and the corners received 1 to 2 lx. Prior to use, feces and urine were removed and each arena was swabbed down with about 10 cc of 80% ethanol solution, and wiped dry with paper toweling. The air-conditioned testing room was maintained at approximately 22°C. The arenas were in a locked room well insulated from outside sounds. Within 1 min of the start of each test the experimenter left the room for the remainder of the automatically recorded 2-h test.

Recording

Horizontal locomotion was recorded using a Topica (model TP-505D/3) CCD video camera and a Sharp (model XA-601) video cassette recorder. Parallax was minimized by mounting the cameras 2 m above the arenas. The 1 m² arena was visualized as 240 × 240 pixels. Therefore, a movement over 24 pixels was a move of 10 cm. A speed of 30 pixels/s was about one rat body length/s, or 7.5 m/min. Raw data recorded in pixels/s were converted to m/min before data analysis was completed. All video records were archived following computer analysis.

Locomotor Analysis

Locomotor activity was quantified using Apple Power Macintosh-based software and a Macintosh (model 7100/80 with an AV board installed) (6,7). The software calculates the

center of mass of the rat. To avoid including the rat's tail in determining the location, or movement; India ink was applied to the tail prior to PB administration. Each 2-h recording was reduced to an ASCII file of observations at 1-s intervals that represented both the positions of the subject on a 240 × 240 pixel grid (X,Y coordinates) and the running average of locomotion rate over five observations. Sampling at 1-s intervals filtered out recording of short-range stereotypic movement that would otherwise have been scored as locomotion. The raw data were used to calculate speeds for each second of the record, which were then used in lieu of the running average provided by the original analysis.

The number of times that the subject was recorded in the center 50% of the arena was filtered so that only those times that the subject was moving faster than 1.2 m/min (2 cm/s) were counted. That filter excluded observations that might have occurred if a subject had become inactive, thereby avoiding a high center zone score for a subject that had collapsed in midarena.

The ASCII file for each subject was then imported into StatView and further analyzed for locomotion rate and thigmotactic response in six 20-min bins of the 2-h test period.

Blood Serum Analysis

An estimate of the serum level of PB in an individual rat at the beginning of the test period was obtained by waiting at least 5 days after a given rat's locomotion test and taking a blood sample by decapitation 30 min following a second similar dose and anesthesia with methoxyflurane. Female subjects were dosed the second time during the appropriate stage of the estrous cycle. Three milliliter blood samples were kept on ice for 2 h, centrifuged, serum drawn off, and frozen at -70°C. The serum was then analyzed for PB as follows: the serum sample was transferred to a stoppered tube and vortexed with 1 ml of 0.025 M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column that have previously been conditioned under vacuum on a Vac Elut manifold (Varian) with methanol, water, and 0.025 phosphate buffer. After application of the sample, the column was air dried for approximately 30 s and then washed with phosphate buffer and 0.1 M acetic acid. The column was again air dried for 30 s before eluting off the adsorbed drugs with 3% ammoniacal methanol. The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 µl of methanol. A 20-µl aliquot of the extract was then used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 ml/min to a Hypersil 5 µm ODS column. A Waters C18 Guard Pak precolumn was used to protect the analytical column. The detector was a Waters 486 variable wavelength detector set at 272 nm with a Dell 486 data system and Millenium (TM) software. The mobile phase consisted of acetonitrile-0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid. 70:30). Quantitative analyses were achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05-5 µg/ml.

Experimental Design

The experimental design was three groups of rats × four application rates × 10 subjects for each application rate. Space limitations in the rat colony required that the rats be tested in two batches, 20 males and 40 females each, for a grand total of 120 rats. Each batch was tested over a 15-22-

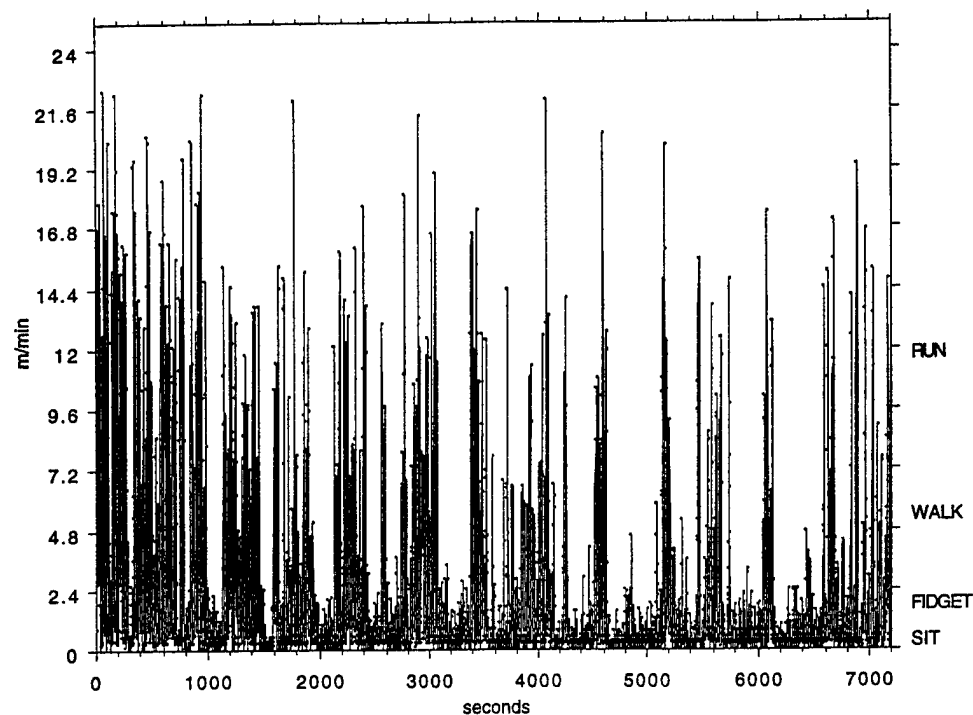


FIG. 1. Typical range and variation in locomotion (m/min) during a 2-h period following administration of vehicle. Note the indications of the type of behavior associated with various speeds at the right margin of the figure.

day period, with 29 days between batches. The data from the two batches were pooled.

Statistical Analysis

Differences between locomotion rates and counts of observations in the center zone of the arena were determined by repeated-measures ANOVA (group \times time), for the total 2 h observation time. Comparisons between groups were then done using Duncan's Multiple Range Test ($p < 0.05$). Subsequently, post hoc power calculations were done with assumptions of higher alpha levels using GPOWER (5).

RESULTS

Locomotion rates as high as 30 m/min were observed. Figure 1 illustrates the range and variation of locomotion rate for a typical rat dosed with vehicle. Sitting, fidgeting, walking, and running fall into the progressively higher ranges indicated in the figure. The ranges of speed associated with these activities were subjectively determined, and are provided as a general indication of the alternation of activities over the observation period. Also, Fig. 1 shows a trend toward fewer and shorter peaks throughout the 2-h period as well as the rapid changes in speed.

Locomotion Rate

Habituation of the locomotion rate, as suggested by the reduced number of peaks over time, is more clearly illustrated by the mean speeds found in each successive 20-min period of observation. Figure 2 shows the habituation curves for all groups and treatments of rats. Each group and treatment followed the same pattern, i.e., a rapid decline in mean speed

during the first hour, followed by very little change in mean speed during the second hour. The dose effect of PB can also be seen in this figure.

Figure 2 shows locomotor activity (speed in mean m/min) during 20-min segments of the experimental session for male rats and female rats in either proestrous or metestrous phase of the estrous cycle following the administration of vehicle or PB. ANOVA revealed a significant three-way interaction among time of observation, dose, and gender, $F(30, 535) = 1.72, p < 0.03$. This figure shows that for subjects given the vehicle speed decreased from an initial high of about 4 m/min to about 1.75 m/min during the final 20 min of the session. ANOVA revealed that the speed decreased as a function of dose, $F(3, 107) = 34.80, p < 0.01$. Post hoc analyses showed no significant differences between the administration of vehicle and 3 mg/kg PB, but that the speeds observed after administration of 10 mg/kg PB and after 30 mg/kg PB were significantly lower than vehicle. Planned contrast analyses at each time of observation (Table 1) showed that there were no significant differences between vehicle and 3 mg/kg PB in any of the groups of subjects. Significant differences were observed at all times of observation when the behavioral effects of vehicle were compared to those observed after administration of 10 mg/kg in metestrous and proestrous females. However, in male rats the decrease in speed after 10 mg/kg PB was only significant at time point 2. Planned contrast analyses showed that speed decreased significantly compared to vehicle administration in all groups of subjects after the administration of 30 mg/kg PB.

Center Zone Activity

The distribution of activity within the 1-m² arena favored the marginal area in all cases. That bias is illustrated in Fig. 3,

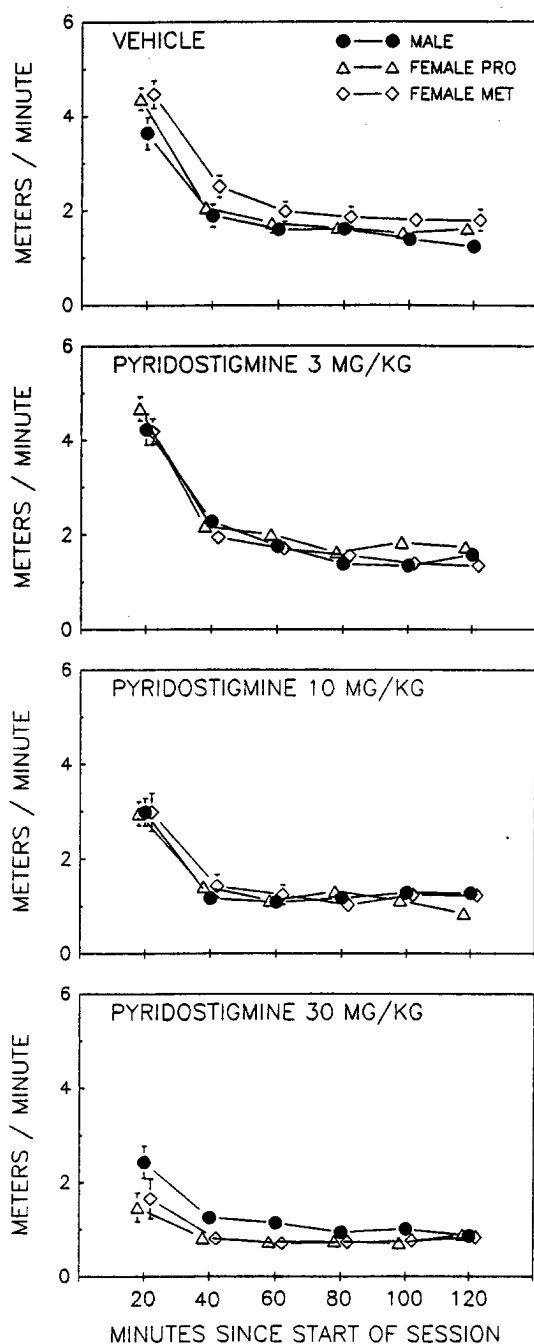


FIG. 2. Mean locomotion rates (m/min) in 20-min segments of 2-h observation periods according to rate of administration of pyridostigmine bromide.

which shows typical traces of the paths of rats given the four treatments used in our study. We quantified the distribution of activity by calculating the percent of the total observations in which the rat was observed moving through the central 50% of the arena. A dose effect was found in all groups of rats.

Figure 4 whose center zone activity for male rats, proestrous female rats, and metestrous female rats following administration of vehicle and 3, 10, or 30 mg/kg PB vs. vehicle.

TABLE 1
EFFECTS OF PYRIDOSTIGMINE BROMIDE (10, 30, AND 10 mg/kg vs. 30 mg/kg) ON LOCOMOTION RATE BY TIME PERIOD OF MALE, AND PROESTROUS AND METESTROUS FEMALE RATS

Pd.	Males			Dose (mg/kg) Proestrous Females			Metestrous Females		
	10	30	10 vs. 30	10	30	10 vs. 30	10	30	10 vs. 30
1	NS	33	NS	28	59	*	36	60	*
2	34	34	NS	28	60	*	50	68	NS
3	NS	NS	NS	30	56	*	37	65	*
4	NS	NS	NS	19	48	*	49	62	NS
5	NS	42	NS	26	41	*	28	57	*
6	NS	30	NS	40	35	NS	33	54	NS
Tot	NS	33	NS	30	50	NS	40	62	*

*Significant effects ($\alpha = 0.05$) are indicated by the percent reduction from the control mean for comparisons in the first two columns. An asterisk indicates a significant difference where the effect of 10 mg/kg vs. 30 mg/kg is compared.

This figure shows that subjects tended to spend between 20 and 25% of the session time in the center of the arena following vehicle administration. PB dose dependently decreased the percentage of center zone observations, $F(3, 107) = 28.85$, $p < 0.01$. After the administration of 30 mg/kg PB subjects were in the center zone in less than 10% of the observations. Gender differences or interactions between dose and gender were not found.

Blood Serum Analyses

Posttreatment analyses indicated that serum levels of PB for the three test groups were higher, but not proportionately

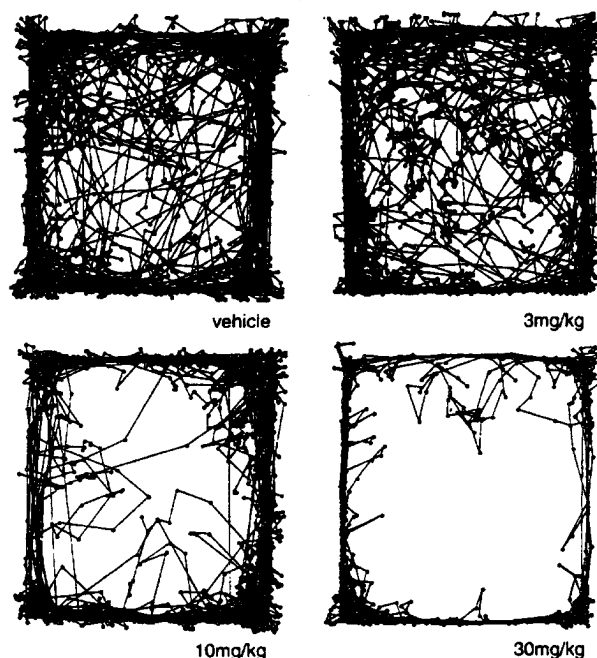


FIG. 3. Typical traces of the paths of male rats during a 2-h observation period, according to the indicated rate of administration of pyridostigmine bromide.

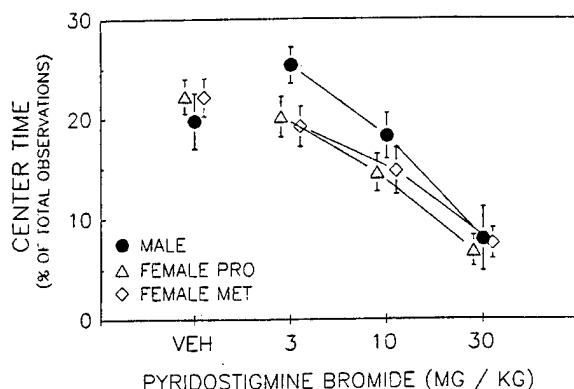


FIG. 4. Center zone time (the average percentage of the total number of observations that the subjects were in the center area (50% of the arena ± 1 SEM) for the groups of male and (proestrous and metestrous) female rats following administration of the vehicle and 3, 10, or 30 mg/kg of pyridostigmine bromide.

higher, with increased dose, i.e., a negatively accelerating dose-response curve. Figure 5 shows serum levels of PB observed 30 min after the second administration of 3, 10, or 30 mg/kg. A total of 70 observations figured in this analysis. No PB was found in control animals. The serum levels differed by dose. A significant interaction between PB dose and gender, $F(4, 61) = 5.64$, $p < 0.01$, and subsequent post hoc analyses supported the observation that PB levels in males differed only when compared after 3 and 30 mg/kg PB. In metestrous females, all three doses differed from one another, whereas in proestrous females differences were observed when 3 and 30 mg/kg and 10 and 30 mg/kg were compared, but not when 3 and 10 mg/kg were compared.

DISCUSSION

We have presented our locomotion data in terms of speed in m/min. The observed speeds correspond to the following types of activity, and provide an illustration of the types of behavior seen. A mean rate of less than 1.2 m/min indicated a sluggish rat moving less than 0.2 body lengths/s. Fidgeting or grooming behavior was recorded as movement less than 2.4 m/min (less than 0.5 body lengths/s). Walking resulted in a mean speed of less than 7.2 m/min (less than 1.5 body lengths/s). Running resulted in speeds ranging from 7.2 m/min to more than three times that rate.

We found gender differences and PB effects on locomotion rate. A previous study on male rats ($n = 6$) found no effect on the running speed in an open-field test following intraperitoneally administered PB at less than 10% of the LD_{50} (14). Recently, hens ($n = 5$) given 5 mg/kg PB orally for 60 days showed no locomotor effects (1). However, both studies lacked the power needed to find anything less than a catastrophic effect. In another study of PB effects on locomotion, male rats administered pyridostigmine and running on a treadmill became exhausted more rapidly than controls (11). Our findings that female rats were more sensitive than male rats, and the somewhat limited power of our test, suggest that additional tests using female rats in numbers adequate to balance type I and type II error are needed to find or rule out subtle effects. The problem of finding effects on sensitive but rare individuals within a population should also be addressed.

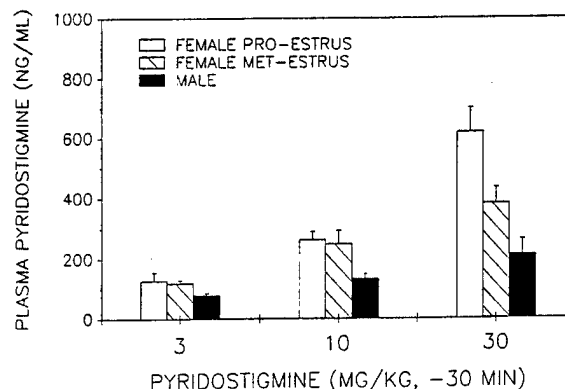


FIG. 5. Serum levels of pyridostigmine bromide (mean nanograms per ml, ± 1 SEM) observed after the second administration of 3 mg/kg (left-most bars), 10 mg/kg (middle bars), or 30 mg/kg (right-most bars).

Center zone activity has been found to be a more sensitive measure of intoxication than speed when a stimulant was the toxicant (3,18). We found that PB depressed both measures, but we are not convinced that one measure is more sensitive than the other in our study. Separating the possible interaction of the two is outside the design of this study.

Serum levels of PB were higher in female rats than in male rats. In female rats they were also higher during the proestrous than during the metestrous phase of the cycle. These observations suggest that PB kinetics (liver metabolism and/or urinary excretion) may be modified by circulating gonadal hormones. At present, it is not known what mechanisms might be involved, but such warrants further investigation.

Table 1 shows the percent reduction from control level of locomotion rates for all cases significant at the 0.05 level. The contrast between the sexes is striking, with little effect observed in males. And, although ANOVA failed to show a significant difference between groups of females, the metestrous females quite consistently showed a greater reduction than the proestrous females.

Sublethal behavioral effects, by definition, are more sensitive and more relevant to drug safety than LD_{50} , or even an LD_1 . The gender effect in rats is certain, with timing relative to estrous cycle a possible exacerbating factor in the toxicity of PB to females. If humans in general, and females at a crucial point in their menstrual cycle in particular, are more sensitive to PB than rats, the changes in rat locomotor behavior that we have found may be relevant to the clinical use of PB.

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**INTERACTIONS OF PYRIDOSTIGMINE BROMIDE, DEET
AND PERMETHRIN ALTER LOCOMOTOR BEHAVIOR OF RATS**

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ORIGINAL RESEARCH

Interactions of Pyridostigmine Bromide, DEET and Permethrin Alter Locomotor Behavior of Rats

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ABSTRACT. Drug interactions have been suggested as a cause of Gulf War Syndrome. Pyridostigmine bromide (PB), a prophylactic treatment against potential nerve gas attack, the insect repellent DEET, and permethrin (PERM) impregnated in soldiers' uniforms may have interacted and caused greater than expected toxicity. We tested those 3 drugs singly and in combinations on male and female Sprague-Dawley rats in open field arenas to find the effects on rate of locomotion and thigmotaxis. Administration rates were 10 mg PB/kg; 50, 200, or 500 mg DEET/kg; 15, 30, or 60 mg PERM/kg; 5 mg PB/kg + 100 mg DEET/kg; 5 mg PB/kg + 15 mg PERM/kg; 100 mg DEET/kg + 15 mg PERM/kg; or vehicle by gavage and ip injection. Locomotor behavior was quantified by video-computer analysis for 2 h post-treatment. Female rats were tested in either pro- or metestrus. Drug interactions were determined by the isobolographic method. Blood serum drug concentrations were estimated by high performance liquid chromatography or gas chromatography-mass spectrometry. Single drug effects were very limited within the ranges tested. Double-drug administrations at half the single-drug rates resulted in statistically significant interactions in male rats for both locomotion rate and thigmotaxis. Combination of PB+PERM and DEET+PERM significantly affected speed, whereas only the combination of DEET+PERM significantly affected thigmotaxis. Female rats did not show significant interactions. Our data suggest that serum concentrations of PB and DEET may have been higher in females than males. Administration of PB+DEET may have reduced the serum concentration of DEET, and administration of PB+PERM may have increased the serum concentration of PERM.

One possible cause of the Gulf War Syndrome is an interaction of 3 drugs that many soldiers may have been exposed to during service in the Persian Gulf War (1-4). These drugs are pyridostigmine bromide (PB) administered as a prophylactic against nerve gas, permethrin (PERM) an insecticide applied to the soldiers' uniforms, and N,N-diethyl-m-toluamide (DEET) an insect repellent used by some soldiers. Pyridostigmine bromide is a cholinesterase inhibitor, and PERM interferes with sodium channels, receptor-ionophore complexes, neurotransmitters, and ATP-ases (5,6), whereas the mode of action of DEET is less well understood (7,8). However, DEET is toxic at high doses, acting as a demyelinating agent which causes spongiform myelinopathy, and is synergistic with other toxicants (2,7,9-12).

The acute toxicity of PB to rats when administered concurrently with PERM or DEET is greater than the expected additive effects (10). The LD50 of various compounds was reduced (toxicity increased) when administered topically and concurrently with DEET to cockroaches (7). The locomotor behavior of chickens was affected by concurrent administration of PB, PERM, and DEET (2). Likewise, the insecticide chlorpyrifos, an acetylcholinesterase inhibitor, has a neurotoxic interaction with PB and DEET (13). The effect of PB alone on rat shuttlebox performance was shown several years ago (14). The learning behavior

of rats was affected by acute administration of PB alone (15). Furthermore, the locomotor behavior of female rats was more depressed than that of males following acute administration of PB (16).

The behavioral effects of PB, DEET, and PERM on humans are not well known. Pyridostigmine bromide administered to 90 volunteers caused limited muscarinic effects and was judged safe and well tolerated (17). However, during the Persian Gulf War, a survey of Israeli soldiers who ingested 30 mg PB every 8 h for only 24 h found many reporting general malaise, dizziness, or imbalance (18). Human exposure to DEET at high rates has been associated with insomnia, mood disturbances, and seizure, but the risk of adversity from label-directed use was judged to be low in 1 review (19). However, a more recent and extensive review concluded that human safety data for DEET are incomplete (20). Occupational exposure to pyrethroid insecticides, including PERM, has been associated with fatigue, but no other behavioral effects have been reported (21). A toxic interaction of DEET and the pyrethroid insecticide fenvalerate has been reported in cats following dermal application (6).

The toxicity of a given compound may be affected by a variety of factors, such as drug interactions, level of stress, age,

sex, route of administration, and genetic makeup. Unexpected interactions of various drugs with grapefruit juice, and varied susceptibility of individual patients illustrate interactions and individual differences as factors in toxicology studies (22). A recent study reported an antagonistic effect of PB on the distribution of PERM to the brain when administered to 4 rats fed PB-impregnated chow versus 5 control rats (23). Pyridostigmine bromide administered to mice stressed by forced swimming caused a 50% reduction in brain acetylcholinesterase activity at less than 1/100 the usual dose (24). A related study found long-lasting changes in acetylcholinesterase activity following stress (25). Age and gender are factors in DEET toxicity to rats and possibly in humans (9). *In vitro* percutaneous movements of pesticides (malathion and glyphosate) from fabric through human skin have been demonstrated (26). Using mouse, rat, and pig skin *in vitro*, recent studies have found that solvent type and concentration in combination with DEET affect the amount of percutaneous absorption of carbamate and pyrethroid compounds (27,28). Monogenetic and polygenetic traits contribute to adverse drug reactions (29) and have been suggested as a factor in variation in the occurrence of Gulf War Syndrome (30). Two human genes have a significant effect on hydrolysis of organophosphate pesticides (31). We have chosen to focus on the interaction of mixtures of chemicals to which soldiers were exposed as a potential factor in Gulf War Syndrome.

Methods of study of the interactions of chemical mixtures have been extensively reviewed (32-34). Isobolographic analysis quantifies and illustrates drug interactions through comparisons of responses to single- versus multiple-drug administrations.

The purpose of this study was to show interactions, if any, of various combinations of PB, DEET and PERM on the locomotor behavior of male and female rats. Synergism of acute toxicity of the drugs in question has been found in rats (10). We chose locomotor measures as responses that maybe more sensitive to drug administration at rates well below a lethal dose, ie rates nearer those to which the soldiers were exposed. Motor activity, an "apical" test of nervous system function (35), has been recommended for determining neurotoxicity (36). In addition, we studied the effects of DEET and PERM alone on rat locomotion, as well as the concentration of PB, DEET and PERM in the blood serum of our test subjects.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (200-250 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed same-sex, 2/cage under a reversed 12 h light-dark cycle in a temperature controlled environment. The rats were identified by ear-punch code. Each rat was handled about 30 sec daily for at least 2 w prior to testing. Treatments were assigned at random within gender categories and time of test. Tests were done between 900 and 1700 h (1500 and 2300 h of the dark phase). Male, and pro- and met-estrus female rats were tested 2 at a time in individual arenas. Male rats were tested first, and whenever possible met-estrus females were tested second and pro-estrus females last, or alternatively only females of 1 estrus type were tested if both types were not available on a given day. All drug administration and handling of test subjects was done by the same technician. The rats were tested in 6 batches of 24 males and 48 females each, plus batches of 12 males and 14 females which received 100 mg DEET/kg +15 mg PERM/kg, for a total of 458 rats. Each

batch was tested over a 18-31 d period, with 19-54 d between batches.

Estrus Stage Determination

Female subjects were examined 1 to 3 h before testing to determine their estrus cycle status. Assignment to pro- or met-estrus categories was based on microscopic examination of epithelial cells found in the vaginal fluid of the rats.

Drugs and Dosage

Pyridostigmine bromide (Sigma Chemical Co, St Louis, MO), was administered in a volume of 5 ml/kg via gavage tube in distilled water at 10 mg/kg or 5 mg/kg when combined with either DEET or PERM. DEET (Sigma Chemical Co, St Louis, MO) was administered by gavage at 50, 200, or 500 mg/kg, or combined with PB at 100 mg/kg. Permethrin (Coulston Products, Easton, PA, via WC McCain), was administered ip in saline-EtOH-Emulphor at 15, 30, or 60 mg/kg, or combined with 15 mg PB/kg. Also, DEET and PERM were administered together at 100 mg DEET/kg and 15 mg PERM/kg. Control animals were dosed with an appropriate volume of mineral oil and distilled water or PERM vehicle. Thirty min after drug administration subjects were placed in the center of the arena about 30 sec prior to recording their activity.

Apparatus

The tests were done in 2 black ABS plastic arenas (100 x 100 x 30 cm high), each surrounded by a black curtain. The arenas were on opposite sides of a rack that supported lights, video cameras and video cassette recorders. Indirect low intensity light was provided by 3-60 Watt red bulbs approximately 2.2 m above each arena and located so that the center of each arena received about 2 lux and the corners received 1 to 2 lux. Prior to use each arena was swabbed down first with water and then with about 10 ml of 70% ethanol solution and wiped dry with paper toweling. The arenas were in a locked air-conditioned room well insulated from outside sounds. Within 1 min of the start of each test the experimenter left the room for the remainder of the test.

Recording

Each test subject was placed in the arena for 2 h, 30 min after PB or DEET administration and 15 min after PERM administration. Horizontal locomotion was recorded using a Topica (model TP-505D/3) CCD video camera and a Sharp (model XA-610) video cassette recorder. The 1 m² arena was visualized as 240 x 240 pixels. Therefore, a movement over 2.4 pixels was a move of 10 cm. Prior to testing, the tail of each rat was blackened with India ink so that only the body was visible to the camera.

Locomotor Analysis

Locomotor activity was quantified using Apple PowerMacintosh-based software (37) and a Macintosh computer (model 7100/80 with an AV board installed). Each 2 h recording was reduced to an ASCII file of observations at 1 sec intervals that represented both the positions of the subject on a 240 x 240 pixel grid (X,Y coordinates) and the running average of locomotion rate over 5 observations. Sampling at 1 sec intervals filtered out recording of short-range stereotypic movement that would otherwise have been scored as locomotion. The raw data was used to calculate speeds for each observation of the record. The automatic aspect of the analysis software resulted in treatment blind analysis.

The number of times that the subject was recorded in the center 50% of the arena was filtered so that only those times that the subject was moving faster than 1.2 m/min (2 cm/s) were counted. That filter excluded observations that might have occurred if a subject had become inactive, thereby avoiding a high center zone score for a subject that had collapsed in mid-arena.

The ASCII file for each subject was then imported into StatView (Abacus Concepts, Berkeley, CA) and further analyzed for locomotion rate and thigmotactic response.

Blood Serum Analyses

Quantitation of the serum level of PB, DEET, or PERM in each rat at the beginning of the test period was obtained by waiting at least 5 d after a given rat's locomotion test and taking a blood sample by decapitation following methoxyflurane anesthesia 30 min after a similar dose. Female subjects were dosed the second time during the appropriate stage of the estrus cycle. Three ml blood samples were kept on ice for 2 h, centrifuged for 15-20 min at approximately 3000 revolutions/min, serum drawn off, and frozen at -70 C.

The serum was analyzed for PB as follows: The serum was vortexed in a stoppered tube with 2 ml of 0.5 M potassium phosphate buffer at pH 10.5. That mixture was then applied to a C18 Prep Sep extraction column (Fisher Scientific p-453) which had previously been conditioned with 5.0 ml methanol and 5.0 ml distilled water. After application of the sample, the column was washed with 5.0 ml of 0.05 M potassium phosphate buffer pH 10.5 and 5.0 ml of methanol. Pyridostigmine bromide was eluted with 3.0 ml of 1% acidic acid in methanol, evaporated to dryness under nitrogen, and the residue reconstituted in 200 μ l of mobile phase A (MP-A). A 50 μ l aliquot was applied to an Ultrasphere Octyl column, 5 microns, 4.6 mm x 25 cm (Beckman Instruments, Fullerton, CA). The high performance liquid chromatograph system consisted of an Hewlett Packard HP 1100 Series Quaternary Pump, HP 1100 Series Thermostatted Column Compartment, HP 1100 Autosampler, HP 1100 Series Vacuum Degasser, HP 1100 Series Variable Wavelength Detector operated at 208 nm, and HP Chemstation for LC Systems software. Mobile phase consisted of low pressure mixing of 2 solvent systems (MP-A and MP-B) at 50% (volume) for each by the 1100 Series Quaternary Pump. MP-A consisted of acetonitrile/water (30:70), 0.1 % sodium lauryl sulfate(wt/v), 0.1% H₃PO₄ (v/v), and tetramethylammonium chloride. MP-B consisted of acetonitrile/water (30:70), 0.4% sodium lauryl sulfate(wt/v), 0.1% H₃PO₄ (v/v). Quantitative analysis was achieved by comparison of peak areas with extracted serum standards over the range of 0.0-600 ng/ml of serum. Flow rate was 1.0 ml/min. Column temperature was maintained at 25 C. The column was flushed with acetonitrile/water (50:50) prior to each day's analysis. The column was equilibrated with mobile phase followed by injection of a PB in water standard range to check for analate response.

The serum samples were analyzed for DEET and PERM as follows: A 200 mg Clean Screen solid phase extraction cartridge (sorbent type CSDAU, Worldwide Monitoring) was conditioned with 2 ml acetone, 2 ml methanol, and 2 ml deionized water. A 0.5 ml sample of serum was transferred to the cartridge reservoir and allowed to percolate by gravity through the sorbent bed. The cartridge was washed with 2 ml deionized water, placed on a vacuum manifold and dried under full vacuum for 5 min. DEET and PERM were eluted from the cartridge with 1 ml of acetone and collected in a graduated conical tube. A 10

μ l volume of internal standard (40 ng/ μ l of US108, Ultra Scientific) was added to the tube, the final volume was adjusted to 1.0 ml and the extract was transferred to a 2 ml GC vial for analysis. The extracts were analyzed for DEET and PERM using a Hewlett Packard 6890 gaschromatograph coupled to a Hewlett Packard 5973 mass selective detector operating in the electron impact mode. The gas chromatograph was equipped with a 30 m HP-5MS column (250 μ m diameter with a 0.25 μ m film thickness) operated in the splitless mode at a flow rate of 0.8 ml/min. A 1 μ l aliquot of the final extract was injected and the analytes were separated using inlet temperature of 275 C and initial oven temperature of 40 C. The oven was ramped at 10 C/min to 270 C and then held at 270 C for 5 min. The final temperature was maintained for 6.8 min. Under these conditions, the retention times for DEET, cis-PERM, and trans-PERM were 17.4 min, 27.6 min, and 27.8 min, respectively. The detector was operated in the selected-ion-monitoring mode with an impact voltage of 70eV and electron multiplier voltage of 2082V. DEET was quantified using ion 190 and confirmed using ions 119 and 191. Both forms of PERM were quantified using ion 183 and confirmed using ions 163 and 165. The internal standard (phenanthrene d 10) was quantified using ion 188.

Experimental Design

The experimental design for the generation of isobolograms was a simplex lattice arrangement (Cornell 1990) that defined 6 drug-dosage combinations for each of the 3 gender categories of rats. More specifically, male rats, met- or pro-estrus female rats in groups of 9 to 18, were administered the following single- or double-drug combinations: 10 mg PB/kg, 200 mg DEET/kg, 30 mg PERM/kg, 5 mg PB/kg + 100 mg DEET/kg, 5 mg PB/kg + 15 mg PERM/kg, and 100 mg DEET/kg + 15 mg PERM/kg. Shown in Figure 1a are the single- and double-drug combinations as represented by the vertices and midpoints of the edges of a 3-drug triangle or simplex. (Overlap in the design resulted in testing 18 rats in each gender category with 10 mg PB/kg.)

Concurrent with the above treatments were vehicle controls and administration of DEET at 50 mg/kg and 500 mg/kg and PERM at 15 mg/kg and 60 mg/kg. The administrations of DEET or PERM alone were to provide data on the effect of extreme rates.

Statistical Analysis

The effects of the 3 drugs, individually and jointly, on locomotion were measured by fitting models of the speed (m/min) and to the proportion of times observed observation in the center zone (called center zone time) values. The form of the model (34) was: Speed (or Center Zone Time) = $b_1PB + b_2DEET + b_3PERM + b_{12}PB \times DEET + b_{13}PB \times PERM + b_{23}DEET \times PERM$, where the coefficients b_1 , b_2 , and b_3 represent the average speeds or center zone times for the individual drugs PB, DEET, and PERM, respectively.

The model above is called a quadratic mixture model (Cornell 34) where the quantities PB, DEET, and PERM in the model's terms represent proportions of the three drugs of each in each single- or double-drug combination. The coefficients b_{12} , b_{13} , and b_{23} of the cross product terms represent measures of non-linear or nonadditive blending between pairs of drugs.

After the fitted model is obtained from the speeds or center zone times, significance tests are performed on the estimates of

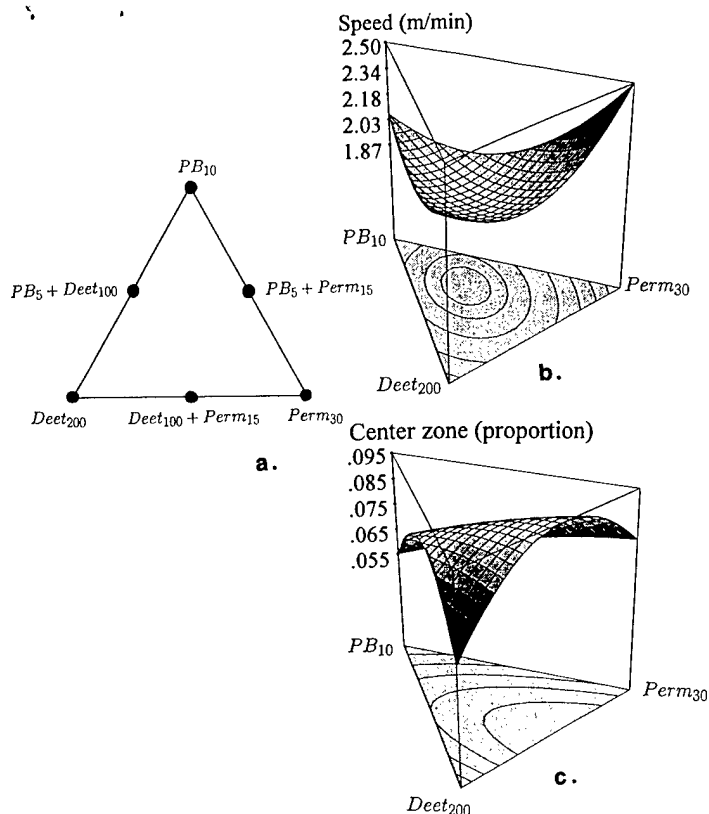


Figure 1. The 3-drug simplex and resultant isobolograms, illustrating a) the drug combinations, b) the isobolgram for male speeds, and c) the isobolgram for male center zone times.

the nonlinear blending coefficients to determine if there is evidence of nonlinear blending (sometimes referred to as synergism or antagonism) between drugs. For visual interpretation of the blending properties of the drugs, the model was used to generate a 3-dimensional plot of the estimated speed or center zone time surface isobologram for the male rats, which is shown in Figure 1a. Means were compared using 2-tailed t-tests, and exact probabilities are reported for all comparisons of blending effects.

RESULTS

Locomotor Effects

Single Drug Effects. Comparisons of all single-drug administrations versus vehicle administrations for all gender categories turned up a significant effect at $P < 0.05$ in only 2/36 cases, ie met-estrus females ($N=9$) that received 500 mg DEET/kg had a reduced speed ($t = -2.207, P=0.042$), and males ($N=9$) that received 60 mg PERM/kg had reduced speed ($t = -2.380, P=0.030$).

Drug Interactions (Blending Effects). The drug interactions were measured utilizing speed and time spent in the center of the arena as outcome measures. A decrease in response resulted in an downward curve in the isobologram. Responses to 3 double-drug combinations can be shown in a single isobologram.

Listed in Table 1 are the average values (m/min) and center zone observations (p of total observations) at the 6 drug treatments for male and female rats. Of interest is the question: "Are the average speed and center zone observations for the double-drug combinations equal to what is expected based on

the additive effects of the single-drugs?" Stated another way, "Is the average speed for the double-drug combination PB 5 + DEET 100 equal to the mean of the average speeds for the individual drugs PB 10 and DEET 200?" If not, is the average of the double-drug combination significantly ($P < 0.05$) higher or lower than the mean of the single-drugs? Listed also in Table 1 are the probabilities or significance levels associated with rejecting the null hypotheses that the value of the double-drug combination equals the sum of the single-drug administrations.

The blending effects of the 3 drugs, singly and in combination, are illustrated by the surface shapes in Figures 1b and 1c for the males and are listed in equation form for the males and females in Table 2. The data points for individual male rats that generated the surface shapes in Figure 1 are shown in Figure 2a-f. The curves in Figure 2 correspond to the curved edges of the surfaces in Figure 1.

The data for pro- and met-estrus females were analyzed separately and then combined. In all 3 cases the blending effects of the drugs on females were strictly additive, resulting in the speed surface and the center zone surface being planar in shape.

With respect to speed (m/min), male rats receiving PB 10, DEET 200, and PERM 30 exhibited speeds of 2.07, 2.24, and 2.50 m/min, respectively (Table 1). These speeds are depicted as the heights of the speed surface directly above the single-drug vertices of the triangle in Figure 1b. When pairs of drugs were administered jointly, the average speeds were 1.95, 1.95, and 2.06 m/min, respectively for PB+DEET, PB+PERM, and DEET+PERM (Table 1). The average speeds of the latter 2 double-drug combinations were significantly lower ($P = 0.026$ and $P = 0.031$) than the mean of the single-drug speeds. In other words, administering PERM+PB or PERM+DEET to male rats resulted in lower average speeds than would be expected due to linear or additive blending of the drugs where these latter speeds are $\frac{1}{2}\text{PERM} + \frac{1}{2}\text{PB} = 2.29$ and $\frac{1}{2}\text{PERM} + \frac{1}{2}\text{DEET} = 2.37$, respectively.

Center zone observations showed that PERM 30 male rats spent more time in the center zone than did male rats adminis-

Table 1. Average speed (m/min) and center zone times (p of observations) at the six drug combinations for male and female rats. Listed below each speed or center zone value is the probability that the double drug average differs from the average of the single drug values when the drugs are additive in their effects.

Response/ sex	Treatment					
	Single-drug			Double-drug		
	PB10	DEET200	PERM30	PB5 + DEET100	PB5 + PERM15	DEET100 + PERM15
Speed/male	2.07	2.24	2.50	1.95 (0.171) ^a	1.95 (0.026) ^b	2.06 (0.031) ^c
C Z /male	0.055	0.062	0.075	0.067 (0.062)	0.068 (0.793)	0.093 (0.033)
Speed/female	1.78	2.20	2.71	2.15 (0.271)	2.06 (0.193)	2.69 (0.200)
C Z /female	0.067	0.070	0.078	0.070 (0.910)	0.080 (0.316)	0.087 (0.233)

Probabilities

^a Reject $[(PB_x/2 + DEET_y/2) = 1/2(PB_x + DEET_y)]$

^b Reject $[(PB_x/2 + PERM_z/2) = 1/2(PB_x + PERM_z)]$

^c Reject $[(DEET_y/2 + PERM_z/2) = 1/2(DEET_y + PERM_z)]$

Table 2. Estimated equations for speed and center zone observations for male and female rats expressed as: speed or center zone = b_1 PB + b_2 DEET + b_3 PERM + b_{12} PB \times DEET + b_{13} PB \times PERM + b_{23} DEET \times PERM.

Response/ sex	Coefficient estimates					
	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}
Speed/male	2.07	2.24	2.50	-0.81 (0.171)*	-1.34 (0.026)	-1.23 (0.031)
C.Z./male	0.055	0.062	0.075	0.067 (0.062)	0.068 (0.793)	0.093 (0.033)
Speed/female	1.76	2.31	2.68	-	-	-
C.Z./female	0.067	0.070	0.078	-	-	-

*Quantities in parentheses represent probabilities of falsely rejecting additive blending of the drugs and inferring nonadditive blending. ($H_0: b_{ij}=0$)

tered PB 10 or DEET 200. Furthermore, male rats administered PB+DEET spent more time in the center zone ($P=0.062$), while male rats administered PERM+DEET spent a significantly ($P=0.033$) greater amount of time in the center zone than was expected based on averaging the single-drug values. (See Fig 1c.)

Blood Serum Drug Concentrations

The following results should be considered in light of the drugs having been administered a second time, that some groups were represented by only 7 samples, and that the PERM analyses were near the limits of our detection method. Therefore, results are presented in Table 3 and Figure 3 without statistical analyses other than an indication of the standard error of the means. However, there were 14-18 samples from groups that received 10 mg PB/kg.

Pyridostigmine Bromide. When administered at 10 mg/kg, PB was found in greater concentrations in the serum of pro- and met-estrus female rats than in males. Figure 3a shows that relationship. However, Figure 3a also shows that when 5 mg PB/kg was administered +15 mg PERM/kg or +100 mg DEET/kg, the concentration of PB was generally about 1/2 to 1/3 that found when 10 mg PB/kg was administered alone, regardless of sex or estrus status.

DEET. When administered at 200 mg/kg, DEET was found at much higher concentrations in the serum of both pro- and met-estrus females than in males (Fig 3b). When DEET was administered at 100 mg/kg + 5 mg PB/kg, considerably less than 1/2 as much DEET was found in each test group (Fig 3b). When administered at 50 or 500 mg/kg, DEET was found in amounts proportional to that found when given at 200 mg/kg for each group of rats (Table 3).

Permethrin. When administered at 30 mg/kg, PERM serum concentration was very similar in all groups (Fig 3c). However, when administered at only 15 mg PERM/kg + 5 mg PB/kg, more PERM was found in the serum of males and females (Fig 3c) than when PERM was administered at 30 mg/kg. When administered at 15 or 60 mg PERM/kg, PERM was found in amounts approximately equal to that found when given at 30 mg/kg for each group of rats (Table 3).

DISCUSSION

The primary goal of this study was to determine if there was other than an additive effect on locomotor activity of double-

drug administrations of PB+DEET, PB+PERM, or DEET+PERM, where the joint amount of each drug was 1/2 the single-drug amount. We found that base-line administration of 200 mg DEET/kg+30 mg PERM/kg had no apparent effect on rate of locomotion or center zone time. However, a previous study had shown that female rat locomotion rate was significantly depressed by 10 mg PB/kg, but not 3 mg PB/kg (16). In this current study we found significant interaction of PB+PERM and DEET+PERM in terms of depressed locomotion rates of male rats when 15 mg PERM/kg was co-administered with PB or DEET at half the rates that produced insignificant effects. Furthermore, administration of 100 mg DEET/kg+15 mg PERM/kg significantly increased center zone time in male rats. These results suggest that both PB and DEET locomotor effects are synergized by PERM, or that PERM locomotor effects are synergized by PB or DEET. Acute locomotor effects on humans were found for PB by others (18) and in several cases for DEET (20).

The lack of any observed interactions in female rats may be the result of a greater PB effect on females and therefore less contrast between single- and double-drug effects, or possible antagonistic effects between PB+PERM or PB+DEET. We found serum PB concentrations higher in female rats than in males, which may explain the failure of females to show an interaction between PB+PERM, as mentioned above. The reported reduced uptake of PERM in the central nervous system (CNS) of rats fed PB (23) may account for this apparent gender difference in locomotion interactions if females take up more PB than males and thus gain greater protection against PERM in the CNS.

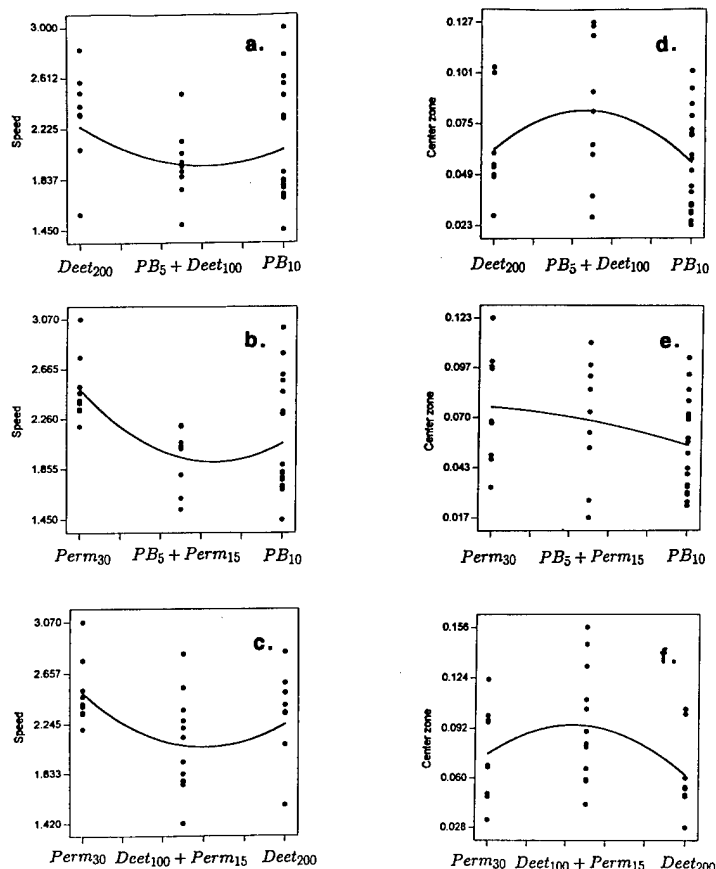


Figure 2. Plots of speed and center zone observations for individual male rats across single- and double-drug treatments. Figures 2a-c correspond to the sides of the 3 dimensional isobologram for speed in Figure 1b. Figures 2d-f correspond to the sides of the isobologram for center zone observations in Figure 1c.

The blood serum concentrations of the drugs used in our study were estimated by a second drug administration several days after the locomotor tests for operational reasons. That forced delay in serum sampling may have allowed enzyme induction, or long-term perturbation of cholinesterase levels may have come into play (25,39). We found serum concentrations of PB somewhat higher in female rats than males. In humans, plasma concentrations were found lower in females than males following acute administration of PB (38), a difference from our results that may be attributable to our repeated administration. In our study, single-drug DEET concentrations appeared higher in females than in males, yet when DEET was combined with PB we found somewhat less DEET than expected. Also, single-drug administration of DEET resulted in proportionally higher serum concentrations with increased administration rate.

In contrast to the serum concentrations of DEET, PERM concentrations were similar in both sexes when administered alone, but were higher than expected in both sexes when co-administered with PB. The PERM blood serum results do not contradict previous findings (23), but may be complementary if PERM is partitioned between blood serum and CNS. Single-drug administration of PERM at increased rates did not result in proportionally higher serum concentrations.

Our results show that immediate behavioral changes result from acute administration of PB+PERM and also after administration of DEET+PERM to male rats. Similarly, chronic administration of those combinations, and others, caused increased neurotoxicity beyond an additive effect in chickens (2,13). Gross toxicity, as measured by LD50's, have shown synergism of these

drug in rats and mice (10-12). Furthermore, the unexpected increase in serum PERM with co-administration of PB in our results may be related to the significantly reduced locomotion rate in male rats given PB+PERM.

The applicability of these results to explaining Gulf War Syndrome is affected by inter-species differences and administration rates. However, small sample sizes, such as we used, and genetic variation in response to cholinesterase inhibitors make finding statistically significant differences difficult. The PB administration rates used in our study were intermediate between the LD50 for male rats and those for soldiers during the Persian Gulf War. Genetic factors in our test population and small sample sizes may have partially masked drug effects. The significant effects that we found justify further investigation and may at least partially explain Gulf War Syndrome.

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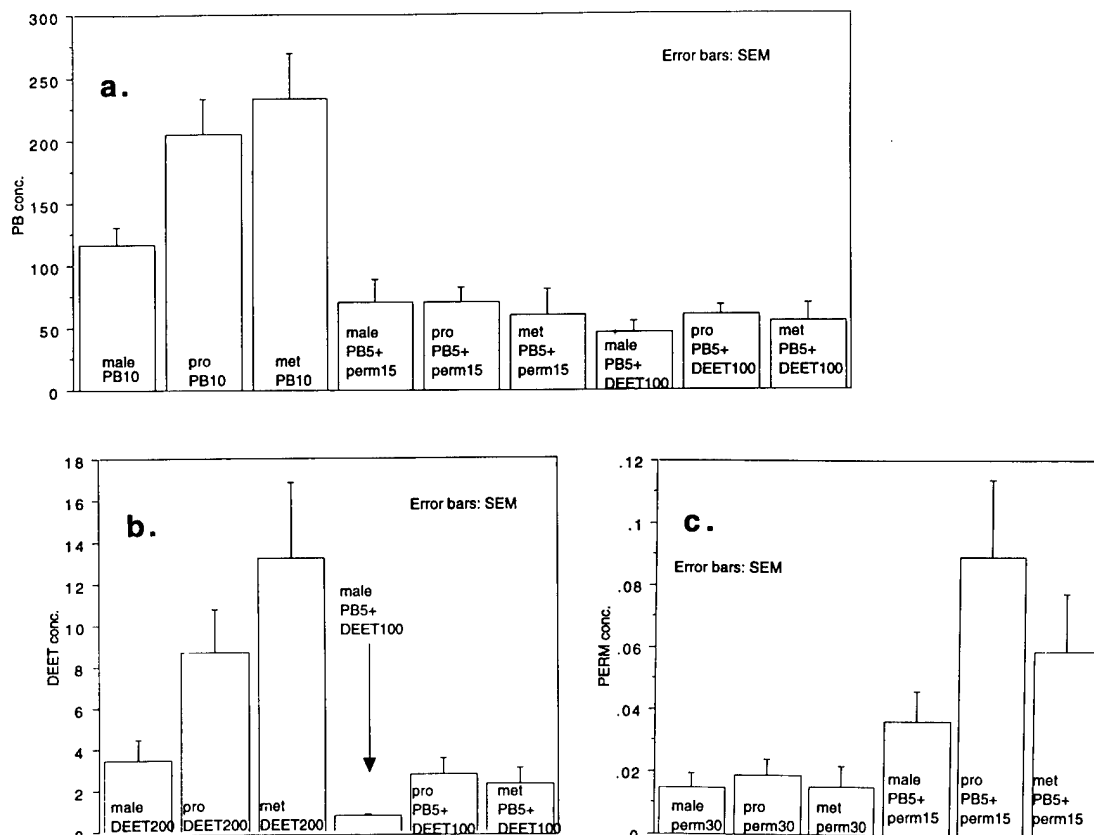


Figure 3. Blood serum concentrations (in ng/ml) of a) pyridostigmine bromide, b) DEET, and c) permethrin, according to drugs administered and gender group. Note that in the cases where 2 drugs were administered only 1/2 the single drug rate was used. Single drug rates were 10 mg PB/kg, 200 mg DEET/kg, and 30 mg PERM/kg.

Table 3. Mean \pm SE of blood serum concentrations of PB, DEET, and PERM in nanograms/ml, according to experimental group, type of analysis, and treatment.

Treatment (N/group)	Group		
	Males	Pro-estrus Females	Met-estrus Females
Analysis for PB			
PB ₁₀ (14-18)	116.2 \pm 14.2	205.4 \pm 28.5	233.8 \pm 35.9
PB ₅ DEET ₁₀₀ (9)	46.6 \pm 8.9	60.0 \pm 8.6	55.6 \pm 14.0
PB ₅ PERM ₁₅ (8-9)	71.0 \pm 17.2	70.6 \pm 11.4	60.2 \pm 20.1
Analysis for DEET			
DEET ₅₀₀ (9-11)	12347 \pm 2181	22392 \pm 5979	23034 \pm 5587
DEET ₂₀₀ (7-9)	3456 \pm 1029	8705 \pm 2029	13237 \pm 3602
DEET ₅₀ (8-9)	1184 \pm 227	5237 \pm 1703	2372 \pm 469
PB ₅ DEET ₁₀₀ (8-9)	733 \pm 147	3549 \pm 1128	2321 \pm 776
Analysis for PERM			
PERM ₆₀ (9)	10 \pm 4	6 \pm 1	11 \pm 3
PERM ₃₀ (8-9)	15 \pm 5	19 \pm 5	15 \pm 6
PERM ₁₅ (9)	18 \pm 6	18 \pm 6	14 \pm 4
PB ₅ PERM ₁₅ (8-9)	42 \pm 10	94 \pm 27	59 \pm 18

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**REPEATED COADMINISTRATIONS OF PYRIDOSTIGMINE BROMIDE,
DEET AND PERMETHRIN ALTER LOCOMOTOR BEHAVIOR OF RATS**

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Repeated Coadministrations of Pyridostigmine Bromide, DEET, and Permethrin Alter Locomotor Behavior of Rats

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ABSTRACT. Interactions of pyridostigmine bromide (PB), permethrin (PERM), and the insect repellent DEET (DEET) have been suggested as possible causes of Gulf War Syndrome (GWS) in humans. Open field locomotor studies have long been used in behavioral toxicology. Using male and female Sprague-Dawley rats, video-computer analyses, and the isobolographic method we have determined the effects on locomotor speed and thigmotaxis following repeated administration of single-, double-, or triple-drug or vehicle controls in an open field. The effects were measured 24 hours after 7 daily drug administrations. Single-drug administrations caused no significant effects. Double-drug administrations resulted in significant effects in the following cases: males given PB + DEET had a significantly slower locomotion rate; males given DEET + PERM had a significantly faster locomotion rate; females given PB + DEET had a significantly slower locomotion rate; and females given PB + PERM spent significantly more time in the center zone (less thigmotaxis). Triple-drug administration caused no significant effect. These results in comparison with behavioral studies in chickens and insects show certain similarities. The implications of the lasting effects on animal models are relevant to GWS in humans.

Gulf War Syndrome (GWS) has been characterized in detail, including various behavioral symptoms (1,2). Great numbers of Persian Gulf War veterans have claimed to have such symptoms (3). Pyridostigmine bromide (PB) was given orally to the troops during the Persian Gulf War as a prophylactic treatment against potential nerve gas attacks and has since been suggested as a possible cause of GWS (4). Pyridostigmine bromide tablets (30 mg) were recommended to be taken 3 times a day (4). A study of 7 male volunteers concluded that chronic PB administration does not impair soldiers' ability to work in a desert environment (5). Following the Persian Gulf War a double-blind study of 90 male and female volunteers given 30 mg PB every 8 h for 21 d found no unexpected side effects (6). However, a survey of Israeli soldiers given 30 mg PB every 8 h for only 24 h found many suffered from general malaise, dizziness or imbalance (7). Three possible explanations for unexpected reactions to PB ingestion are that female troops may differ from males troops in response that individuals may vary in their response, or that synergistic reactions may result from combinations of insect repellents, insecticides, and PB (4). Alternative explanations include interaction of PB and stress or adrenaline (7-9).

The troops were given insect repellent (DEET) and uniforms treated with permethrin (PERM), a chemical that has both repellent and insecticidal action (10). A recent review of the safety of DEET found reports of behavioral effects including ataxia, tremors and convulsions following various use patterns (11). Furthermore, DEET interferes with ammonia metabolism and puts female carriers of ornithine carbamoyl transferase deficiency at increased risk if exposed to DEET (11).

Pesticides can be adsorbed from fabric that is in contact with the skin (12). However, PERM was not carried across the skin barrier by DEET when tested in vitro in pigs and mice (13). PERM is a neurotoxin that affects sodium channels and also inhibits the mitochondrial complex I (14). Coadministration of PB, DEET, and PERM has been shown synergistic in chickens, cockroaches, rats and mice (9,15-17). Based on a very small sample, an antagonism between PERM and PB was reported in rats (18).

Open field locomotion studies have been used in behavioral neurotoxicology for many years (19-23). Recent development of video-computer methods have made analysis of open field behavior easier and more precise than in the past (24).

Analysis of drug interactions can be achieved using the isobolographic method (25-27). The method compares responses to multiple-drug administrations vs single-drug administrations of proportionally higher concentrations. Simultaneous comparison of the interactions of 3 drugs results in a 3-dimensional response surface (isobologram).

The purpose of this study was to examine the effects of repeated administration of PB, DEET, and PERM, alone and in combinations, on rat locomotion rate and thigmotaxis, with consideration for gender.

MATERIALS AND METHODS

Subjects

Sprague-Dawley rats (200-250 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed same-sex, 2/cage

under reversed 12 h light-dark cycles in a temperature controlled environment. Each rat was identified by ear-punch code and handled about 30 sec daily for at least 2 w prior to testing. Treatments were assigned at random within gender categories and time of test.

Tests were done between 900 and 1700 h (1500 and 2300 h of the dark phase). Rats were tested for 1 h, 2 at a time in individual arenas: 4 males followed by 4 females each day. All drug administration and handling of test subjects was done by the same technician. The rats were tested in 3 batches of 32 males and 32 females each. (Each batch was tested over a 8-15 d period, with 14-28 d between batches.) Prior to testing, each subject's tail was darkened with India ink to make the video image of the rat more compact.

Drugs and Dosages

Pyridostigmine bromide, (Sigma Chemical Co, St Louis, MO) was administered in a volume of 5 ml/kg via gavage tube in distilled water at 7.5 mg PB/kg or at 3.75 mg PB/kg when combined with DEET or PERM, or at 2.5 mg PB/kg when combined with both DEET and PERM. DEET (Sigma Chemical Co, St Louis, MO) was administered by gavage at 200 mg DEET/kg, or at 100 mg DEET/kg when combined with PB or PERM, or at 67 mg DEET/kg when combined with both PB and PERM. PERM (Coulston Products, Easton, PA, via W C McCain) was administered ip at 60 mg PERM/kg, or at 30 mg PERM/kg when combined with PB or with DEET, or at 20 mg PERM/kg when combined with both PB and DEET. Each drug was administered by the original route when combined with other drugs.

Control animals were dosed with an appropriate volume of mineral oil and distilled water. Each test subject was held 24 h after the last of 7 daily drug administrations, then placed in the center of the arena about 30 sec prior to the recording of its activity.

Apparatus

The tests were done in 2 black ABS plastic arenas 100 X 100 X 30 cm high, each surrounded by a black curtain. Indirect light from 3 60 Watt red bulbs approximately 2.2 m above each arena provided 1-2 lux on the arena floor. Prior to use, each arena was swabbed first with water and then with about 10 ml of 70% ethanol solution and wiped dry with paper toweling. The arenas were in a locked air-conditioned room insulated from outside sounds. Horizontal locomotion was recorded using a topica (model TP-505D/3) CCd video camera and a Sharp (model XA-610) video cassette recorder. The arenas were visualized as 240 X 240 pixels.

Locomotor Analysis

Locomotor activity was quantified using Apple PowerMacintosh-based software (28) and a Macintosh (model 7100/80 with an AV board installed). Each 1 h test was reduced to an ASCII file of observations at 1 sec intervals that represented the positions of the subject on a 240 X 240 pixel grid (X,Y coordinates). Sampling at 1 s intervals filtered out short-range stereotypic movement that would otherwise have been scored as locomotion. The raw data was used to calculate speeds for each observation of the record. The automatic aspect of the analysis software resulted in "treatment blind" analysis. Concurrent with real-time analysis, a tape recording was made for archival purposes.

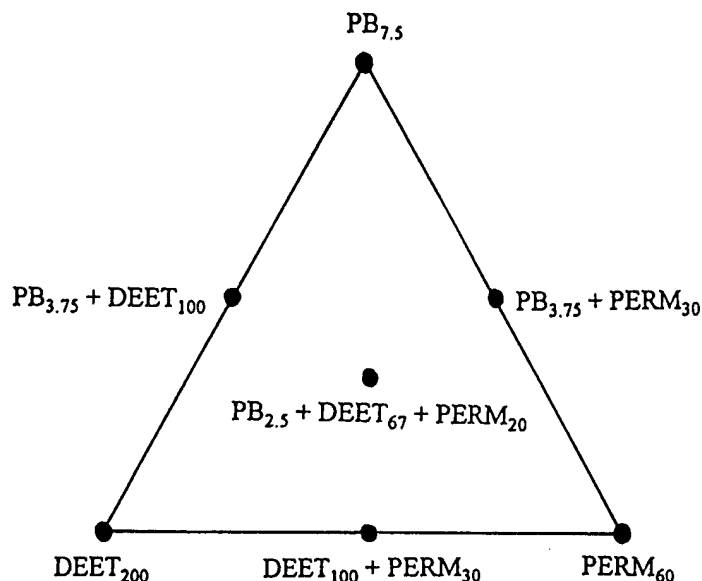


Figure 1. Single- and multiple-drug combinations as represented by the vertices, center, and midpoints of the edges of a 3-drug simplex.

The number of times that the subject was recorded in the center 50% of the arena was filtered so that only those times that the subject was moving faster than 1.2 m/min (2 cm/sec) were counted. The filter excluded observations that might have occurred if a subject had become inactive, thereby avoiding a high center zone score for a subject that had collapsed in mid-arena.

Experimental Design

The experimental design for the generation of isobolograms was a simplex lattice (27) arrangement that defined 7 drug-dosage combinations for each gender of rats in groups of 12. Administered were the following single- or multiple-drug combinations: PB only at 7.5 mg/kg; DEET only at 200 mg/kg; PERM only at 60 mg/kg; PB at 3.75 mg/kg + DEET at 100 mg/kg; PB at 3.75 mg/kg + PERM at 30 mg/kg; DEET at 100 mg/kg + PERM at 30 mg/kg; PB at 2.5 mg/kg, + DEET at 67 mg/kg + PERM at 20 mg/kg. Shown in Fig 1 are the single- and multiple-drug combinations as represented by the vertices, center and midpoints of the edges of a 3-drug triangle or simplex.

The experimental designs for generation of isobolograms for each sex was 7 administration rates X 12 animals/rate of administration. Concurrent with the above treatments were vehicle controls.

Statistical Analysis

The effects of the 3 drugs, individually and jointly, on locomotion were measured by fitting models expressing the speed (m/min) and the time-in-center zone (p of observations) values as a function of the drug proportions. The form of the model (28) was $\text{Speed (or Center Time)} = b_1 \text{ PB} + b_2 \text{ DEET} + b_3 \text{ PERM} + b_{12} \text{ PB} \times \text{DEET} + b_{13} \text{ PB} \times \text{PERM} + b_{23} \text{ DEET} \times \text{PERM} + b_{123} \text{ PB} \times \text{DEET} \times \text{PERM}$, where the coefficients b_1 , b_2 , and b_3 represent the average speeds or center zone times for the individual drugs PB, DEET, and PERM, respectively.

The model above is called a special-cubic mixture model (27) where the quantities PB, DEET and PERM in the model's terms

represent proportions of each of the 3 drugs in each single-, double-, or triple-drug combination. The coefficients b , b , b , and b of the crossproduct terms represent measures of nonlinear or nonadditive blending among the drugs.

After the fitted model is obtained from the speeds or center zone times, significance tests are performed on the estimates of the nonlinear blending coefficients to determine if there is evidence of nonlinear blending (sometimes referred to as synergism or antagonism) between drugs. For visual interpretation of the blending properties of the drugs, the model was used to generate 3-dimensional plots (isobolograms, ie Fig 3) of the estimated speed or center zone time surfaces.

Means were compared using two-tailed t-tests, and probabilities are reported for all comparisons of blending effects.

RESULTS

Single-Drug Effects

No administration of a single-drug, either to males or females, caused a statistically significant lasting effect on speed or center zone time when compared with administration of the vehicle. Of the 12 cases comparing the 2 sexes, 2 measures, and 3 drugs, PERM caused the most variable results, in terms of speed and center zone time. However, none of those cases were significantly different from the vehicle means.

Double-Drug Effects (Blending Effects)

The lasting effects of double-drug administrations on both speed and center zone time are shown in Figs 2 and 3. Figure 2 provides the range of effect, in terms of the 95% confidence limits for the means of 12 subjects that received each double-drug treatment. The single-drug effects are also included for comparison. Figure 3 displays the estimated surfaces generated by models multiple fitted to the data in Table 1 as represented by isobolograms (response surfaces) that illustrate non-additive interactions of the multiple-drug administrations. Table 1 provides the values used to generate the isobolograms and the probabilities of significance.

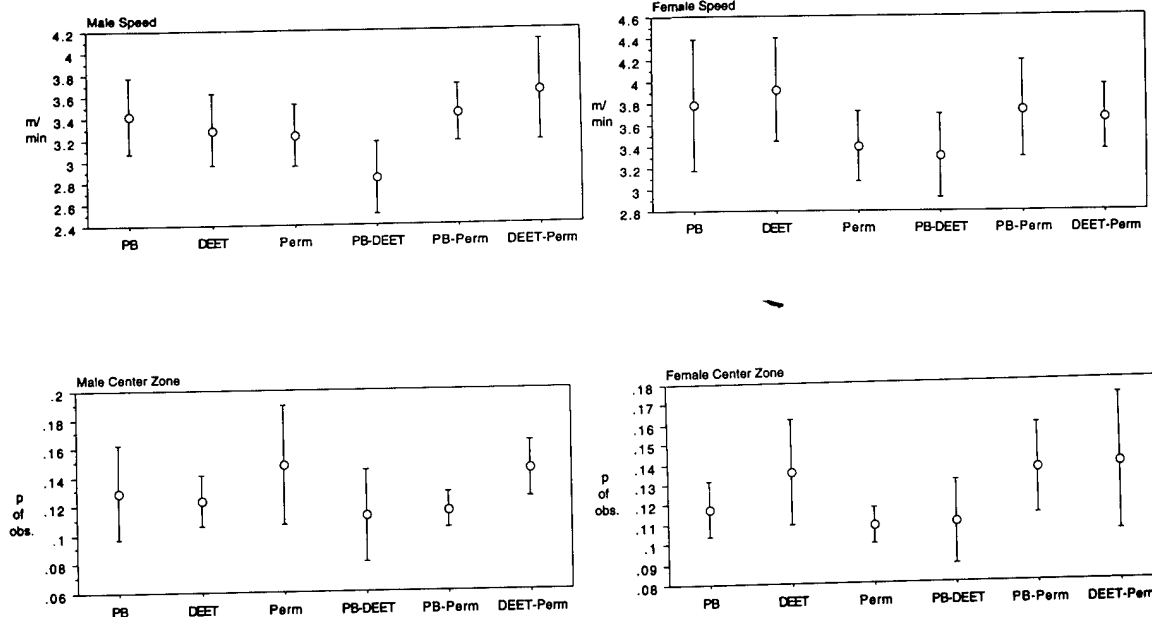


Figure 2. Double-drug effects on speeds and center zone times, with 95% confidence limits (N = 12). Single-drug effects are included for comparison.

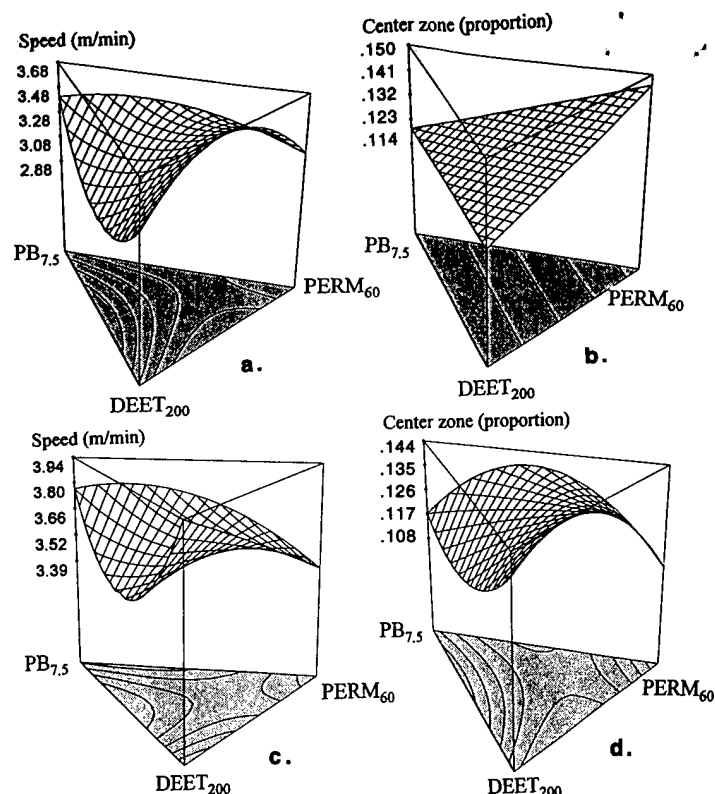


Figure 3. Isobolograms for rat speeds and center zone times, as affected by 7 treatments: a=male speeds; b=male center zone times; c=female speeds; d=female center zone times. See text and Fig 1 for drug administration rates.

Male rats given PB + DEET had a significantly lower speed ($p = 0.022$) than would be expected by averaging speeds for PB and DEET. The lower speed is depicted by the depressed curve of the isobologram surface above the side of the triangle between the PB and DEET vertices (Fig 3a). The rats given DEET + PERM had a significantly higher speed ($p = 0.043$), as depicted by the upward curve of the surface above the DEET and PERM edge. There was no significant nonplanar effect of any combination of drugs on the center zone times for male rats, as depicted by the planar surface shown in Fig 3b.

Table 1. The behavioral responses of male and female rats according to administrations of pyridostigmine bromide, DEET and permethrin, singly and in various combinations. Average speed (m/min) and center zone (cz) times (p of observations) at the single-, double-, and triple-drug combinations for male and female rats. Listed in parentheses below each speed or cz statistic is the probability of detecting nonlinear blending between pairs of drugs when the drugs are additive in their effects in those cases where the probability reached or approached the 5% level of significance. None of the triple-drug exposures were significantly effected.

Sex/ Response	Treatment ^a						
	PB _{7.5}	DEET ₂₀₀	PERM ₆₀	PB _{3.75} + DEET ₁₀₀	PB _{3.75} + PERM ₃₀	DEET ₁₀₀ + PERM ₃₀	PB _{2.5} + DEET ₉₇ + PERM ₂₀
Male/Speed	3.42	3.29	3.23	2.84 (0.022) ^b	3.44 (ns) ^c	3.64 (0.043) ^d	3.46 (ns)
Male/C.Z	0.132	0.122	0.148	0.113 (ns)	0.116 (ns)	0.139 (ns)	0.136 (ns)
Female/Speed	3.78	3.95	3.40	3.37 (0.030)	3.74 (ns)	3.66 (ns)	3.62 (ns)
Female/C.Z.	0.118	0.136	0.109	0.108 (ns)	0.136 (0.023)	0.139 (0.088)	0.139 (ns)

^a Acronyms for the drugs are: pyridostigmine bromide=PB; DEET=DEET; permethrin=PERM and subscripts for each drug indicate dosages in mg/kg.

Probabilities: ^b Reject [(PBx/2 + DEETy/2) = 1/2(PBx + DEETy)]

^c Reject [(PBx/2 + PERMz/2) = 1/2(PBx + PERMz)]

^d Reject [(DEETy/2 + PERMz/2) = 1/2(DEETy + PERMz)]

Female rats given PB + DEET had a significantly lower speed than would be expected ($p=0.030$), as depicted by the depressed curve of the isobologram surface along the edge connecting the PB and DEET vertices (Fig 3c). That effect was similar to the response in males. Unlike the males, there was no significant effect from DEET + PERM.

Female rats given PB + PERM had a significant increase in center zone time ($p=0.023$), as depicted by the upward curve of the surface along the PB-PERM edge of the triangle in Fig 3d. Female rats given DEET + PERM had a tendency ($p=0.088$) for increased center zone time, as indicated by the upward curve of the surface along the DEET-PERM edge.

Triple-Drug Effects (Blending Effects)

In no case where PB + DEET + PERM was administered (at 1/3 the single-drug rates) was a significant effect found. The central point in the isobologram for female center zone time (Fig 3d) is somewhat depressed, in keeping with the lack of significant effect, notwithstanding the upward curves above the PB-PERM and DEET-PERM edges of the triangle.

DISCUSSION

Significant Lasting Interaction

Various combinations of drugs caused lasting effects on speed or center zone time in both male and female rats, despite no significant effects from single-drug administrations at twice the double-drug rates. PB + DEET caused a significant lasting decrease in male speeds, possibly because of greater uptake of PB in combination with DEET (15). PB + PERM caused a significant lasting increase in male speeds. PB + DEET caused a significant lasting decrease in female speeds, possibly because of greater uptake of PB, as in the males. Furthermore,

DEET + PERM caused a significant lasting increase in female center zone times, and a similar tendency was observed for the PB + PERM combination. A recent study (29) found that Wistar-Kyoto rats given PB for 7 consecutive days exhibited exaggerated startle responses as late as 22 d post-dosing.

Comparison with Acute Mixture Results

Male rats given a single administration of either PB + PERM or DEET + PERM had significant reductions in speed (31). The speeds of male rats given repeated administrations differed from those given a single administration for all possible combination; ie a significant decrease for PB + DEET and a significant increase for DEET + PERM, but no apparent effect for PB + PERM. Acute administration of DEET + PERM to male rats caused a significant increase in center zone time (31). However, repeated administrations had no apparent lasting effect on male center zone times.

Acute administration to female rats showed no apparent interactive effects on speed or center zone time, perhaps because of greater overall drug sensitivity than in males (31,32). However, repeated administration of similar drug combinations to females caused significant lasting effects in speeds and center zone times; ie PB + DEET causing a decrease in speed, PB + PERM causing a significant increase in center zone time, and DEET + PERM possibly causing a similar increase. In the latter case, significant at the 8.8% level.

Comparison with Effects in Other Species

The lasting behavioral effects of PB + DEET may well be cholinergic effects resulting from greater uptake of PB, even at half the single-drug administration rate (15). The observed behavioral effect of the interactions of PERM + PB or DEET are less easily explained. Pyrethroids, and PERM specifically, are reported to cause excitability and/or aggressive behavior in vertebrates (29,31-32), and hyperactivity and increased center zone time in insects (33).

Abou-Donia and coworkers (15) reported that repeated coadministration of PB + DEET, or/+ PERM to hens caused locomotor dysfunction, with onset at about 10 to 15 d into a 60 d test. Triple-drug administration caused the greatest overall effect, but administrations were combined single-drug rates, as opposed to one-third single-drug rates as in our study. Furthermore, DEET and PERM were administered sc and at considerably higher rates than in our study. Their hypothesized increased "effective concentration" of DEET and PERM is in keeping with our findings of either DEET or PERM involvement in every significant interaction.

Alzogaray and coworkers (33) found that nymphs of *Triatoma infestans* were more active when exposed to a surface treated with PERM, and that topical administration caused decreased thigmotaxis. We found an increase in center zone time (inhibition of thigmotaxis) in female rats given PB + PERM, and possibly DEET + PERM. Although there may be a confounding interaction between locomotion rate and center zone time, PERM clearly has behavioral effects on a range of species.

Implications of PB, DEET and PERM Interactions

Our results indicate that significant lasting behavioral effects (at least for 24 h) can be caused by repeated administrations and subsequent interactions of PB, DEET and PERM at rela-

tively low coadministration rates, and at rates below single-drug rates that have no apparent effects. The lasting aspect is perhaps the most important consideration if these results are a partial explanation of GWS in humans.

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The Effects of Acute and Repeated Pyridostigmine Bromide Administration on Response Acquisition with Immediate and Delayed Reinforcement

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*Department of Psychology, University of Florida, Gainesville, FL, †Department of Psychopharmacology, Utrecht University, Utrecht, The Netherlands, and ‡Department of Pharmacy Practice, §Department of Environmental Engineering, ¶Department of Medicinal Chemistry, University of Florida, Gainesville, FL 32611-2250

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VAN HAAREN, F., R. DE JONGH, J. B. HOY, J. L. KARLIX, C. J. SCHMIDT, I. R. TEBBETT AND D. WIELBO. *The effects of acute and repeated pyridostigmine bromide administration on response acquisition with immediate and delayed reinforcement.* PHARMACOL BIOCHEM BEHAV 62(2) 389–394, 1999.—This experiment was designed to assess the effects of acute and repeated administration of pyridostigmine bromide (a carbamate with prophylactic and therapeutic uses) on response acquisition. Experimentally naïve, male Sprague–Dawley rats were exposed to a situation in which lever presses were either immediately followed by food-pellet presentation or after a 16-s resetting delay. Different groups of rats received either one acute administration of pyridostigmine bromide (10 mg/kg, by gavage) or repeated pyridostigmine administration for 7 days (1.5 mg/kg/day, by gavage). Other groups were treated with distilled water for the same period of time. Both acute and repeated pyridostigmine bromide administration decreased serum cholinesterase levels by approximately 50%, but neither treatment affected brain cholinesterase levels in our assay. Acute and repeated drug administration produced the same behavioral effects. Subjects exposed to the 0-s delay conditions obtained many more food pellets than those exposed to the 16-s delay conditions. Administration of pyridostigmine bromide delayed the onset of responding in some, but not all, of the subjects in the treated groups, independent of the delay condition to which they were exposed. Many more responses were observed on an inoperative lever during the 16-s delay conditions than during the 0-s delay conditions, especially during the 16-s delay condition in which subjects had received acute vehicle administration. Whether or not these effects of small doses of pyridostigmine bromide on response acquisition are of central or peripheral origin will need to be determined in future studies, as response acquisition in the present experiment may have been affected by pyridostigmine's effects on gastrointestinal functioning and/or motor activity. © 1999 Elsevier Science Inc.

Gulf War Syndrome	Pyridostigmine bromide	Cholinesterase inhibition	Response acquisition
Delayed reinforcement	Lever press	Male rats	

PYRIDOSTIGMINE bromide (PB), a quaternary carbamate, is a reversible inhibitor of acetylcholinesterase (AChE), thereby causing acetylcholine (ACh) to accumulate at receptor sites (18). PB is used in the treatment of myasthenia gravis (7), and as pretreatment under threat of chemical warfare because of its protective effect against organophosphorus (OP)

nerve gases (3,5). OP agents exert their effect by irreversibly inactivating AChE resulting in signs and symptoms consistent with excess cholinergic stimulation. PB protects against OP poisoning by shielding AChE through reversible inhibition of the enzyme in the peripheral nervous system [cf. (1,2)]. Spontaneous decarbamylation occurs following treatment with PB

restoring the activity of AChE (20). PB was taken prophylactically by an estimated 250,000 soldiers during the Gulf War.

Evidence has been presented to show that small amounts of PB are behaviorally active after acute administration. Wolthuis and Vanwersch (21) reported in 1984 that intraperitoneally (IP) administered PB interfered with two-way shuttlebox-avoidance learning, open-field behavior, and complex coordinated movements in rats, without producing overt symptoms and without affecting running speed and simple coordinated locomotion. Similarly, Shih et al. (15) found that low doses (6 and 12 mg/kg) of orally administered PB produced a decrement in operant responding maintained under a multiple fixed-ratio, time-out (multi-FR-TO) schedule of water reinforcement. Consistent with these results is a study by Liu (12), who showed that low doses (3–12 mg/kg) of orally administered PB dose dependently decreased the rate of responding for water reinforcement in a visual intensity discrimination task, again without producing signs of overt toxicity. PB also dose dependently decreased unconditioned water intake in water-deprived rats, but did not significantly affect locomotor activity. On the basis of these results, the author suggested that the disruptive effects of PB on the performance in the simple light intensity discrimination task involved motivational dysfunction rather than motor impairment. However, Hoy et al. (8) have recently presented evidence to show that acute PB administration in the range of that investigated by Liu (12) dose dependently decreased spontaneous locomotor activity in male and, even more so, in female Sprague-Dawley rats.

The present experiment is one of several designed to assess the effects of repeated PB administration on the acquisition of a novel response (learning) in rats. Previous experiments have shown that food-deprived, but magazine-trained, rats will quickly learn to contact a lever in an operant chamber. They will continue to contact the lever at high rates when lever contacts are followed by food presentation (11,19). This paradigm has proven useful to assess the effects of a pharmacological challenge on the acquisition of a new response, thereby providing important information on response acquisition that cannot be derived from assessing drug effects on well-established performance. For instance, Stolerman (16,17) has reported that chlorpromazine and chlordiazepoxide impaired response acquisition when lever presses were immediately followed by pellet presentation. More recently, LeSage et al. (11) have presented evidence to show that rats learn to press a lever following *d*-amphetamine (*d*-AMPH) administration both when pellet presentation occurs immediately following the response or after the expiration of a resetting delay. Differential responding on the operative lever (an index of acquisition) was not affected by *d*-AMPH, however, which led the authors to conclude that this compound did not disrupt response acquisition, except at doses that produced a general disruption in behavior.

The present experiment was designed to assess the effects of acute and repeated PB administration on the acquisition of a lever press response when lever presses were either immediately followed by pellet presentation (delay 0-s) or after the expiration of a 16-s resetting delay (resetting delay 16-s). Previous studies have suggested that the detrimental behavioral effects of drugs or toxins may be more easily recognized under the latter conditions (11). Adult male rats either received one acute administration of a small dose of PB or they were treated with PB for 7 days prior to the acquisition session. The latter treatment conditions (1.5 mg/kg/day) approximated those of the Gulf War, during which soldiers sometimes were ordered to take 3×30 mg PB/day/70 kg for 1 or 2 weeks (9).

METHOD

Subjects

Forty-eight experimentally naïve male Sprague-Dawley rats were obtained from a commercial supplier (Harlan-Sprague-Dawley, Indianapolis, IN) when they weighed between 250–275 g. They were housed in groups of three under a reversed 12-h light-dark cycle (lights on 1800 h), in a temperature- and humidity-controlled environment. The rats were handled daily for 2 weeks before the beginning of the experiment. Standard rodent chow was available in the home cages during the first week. Starting with the second week, home cage rodent chow was limited to approximately 16 g per rat per day, delivered at approximately 1600 h. Water was continuously available in the home cage.

Apparatus

The experiments were conducted in six rodent operant conditioning chambers (Coulbourn Instruments, Allentown, PA). The chambers were 25 cm wide, 30 cm long, and 29 cm high. The side walls were made of Plexiglas and the intelligence panel and the back wall consisted of modular stainless steel panels. The floor consisted of 16 rods, spaced 1.75 cm apart. A pellet tray was located 1.7 cm above the floor in the middle of the intelligence panel, and a houselight was approximately 3 cm from the ceiling of the chamber. The pellet tray could be illuminated during pellet presentation (Noyes, 45 mg rodent purified formula). There were two retractable levers, one to the right and one to the left of the pellet tray. They were spaced 12.5 cm apart and located 6.3 cm above the floor. The levers protruded 1.8 cm from the intelligence panel. Each chamber was enclosed in a sound-attenuating and ventilated cubicle. Experimental events were controlled and data were collected using an IBM compatible computer (GatorByte, Gainesville, FL) with L2T2 software and LabLinc interfacing obtained from Coulbourn Instruments (Allentown, PA).

Procedure

Groups of six rats were exposed to one of eight different experimental conditions. The delay of reinforcement was either 0 s (delay 0-s) or 16 s (delay 16-s resetting). The drugs were administered either acutely or repeatedly, and the rats received either PB or distilled water (PB vehicle). When the drugs were administered acutely, the rats were first trained to retrieve food pellets from the tray in the operant chamber (magazine training). During magazine training, the rats were first placed in the darkened operant chamber and both levers were retracted from the chamber. After 5 min, the houselight was illuminated and pellets were delivered on a variable time (VT) 60-s schedule. Both levers remained retracted during the magazine training session, which was terminated after 60 pellets had been delivered. Subsequently, the rats received distilled water by gavage for 2 days. They were tested 30 min following PB or vehicle administration on day 3. When the drugs were administered repeatedly, the rats were also first trained to eat from the pellet tray. Then, for 7 days, they received either PB or distilled water by gavage, and they were tested 30 min after drug or vehicle administration on day 7.

The acquisition session (which started at 1600 h to include the final 2 h of the subject's dark period) also began with a 5-min dark period, during which the levers were retracted from the chamber. Then, the houselight was illuminated and both levers were extended into the operant chamber. Pressing the left (operative) lever immediately resulted in pellet pre-

sentation during the 0-s delay condition. In the delay condition, pressing the left lever resulted in pellet presentation after 16 s, but only if the subject did not press the lever during the (unsigned) delay interval. A press on the left lever during the delay reinitiated the delay interval. In both conditions, pressing the right (inoperative) lever had no scheduled consequences. The experimental session was terminated after 8 h and the rats were removed from the experimental chamber and returned to the home cage at that time. The data for the different groups of subjects were collected on consecutive days.

Drugs

Pyridostigmine bromide (PB, Sigma Chemical Co., St. Louis, MO) was dissolved in distilled water, and both PB and distilled water were administered by gavage, in a volume of 5 ml/kg. PB was either administered at 10 mg/kg, 30 min prior to the beginning of the experimental session (acute administration) or at 1.5 mg/kg for 7 days at approximately 30 min prior to the scheduled starting time of the experimental session on day 7 (repeated administration).

Serum Preparation

Trunk blood was collected from the six PB-treated rats and the six control rats that participated in the repeated-administration experiment. They received one more administration of PB or vehicle on the day after the response acquisition session 30 min prior to blood collection. The trunk blood of rats who received one acute administration of PB or distilled water was obtained from a group of subjects that had not participated in the response acquisition session, but that, otherwise, had been treated in a manner identical to that of the subjects who participated in the experiment. To collect blood, the rat was placed in a jar containing a paper towel saturated with Metofane (Methoxyflurane), 30 min after PB or vehicle administration. The anesthetized animal was quickly decapitated after 1 min. Blood was collected in a 15-ml polystyrene culture test tube and allowed to coagulate on ice for 2 h. It was then centrifuged for 15–20 min at approximately 3000 revolutions per minute. The serum was then drawn off the solid cell matter with a clean glass Pasteur pipette and placed in a 1.5-ml polystyrene microcentrifuge tube. It was then immediately placed in a freezer (at -20°C) where it was stored for up to 3 months until analysis. Brains were also removed at the time of decapitation, quickly frozen, and stored in the freezer.

Serum Analyses

Pyridostigmine bromide. The serum sample (0.5 ml) was transferred to a stoppered tube and vortexed with 1 ml of 0.025 M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column that had previously been conditioned under vacuum on a Vac Elut manifold (Analytichem) with methanol (2 ml), water (1 ml), and 0.25 M phosphate buffer (1 ml). After application of the sample, the column was air dried for approximately 30 s and then washed with phosphate buffer (1 ml) and 0.1 M acetic acid. The column was again air dried for 30 s before eluting off the adsorbed drugs with ammoniacal methanol (3%, 2 ml). The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 μl of methanol. A 20- μl aliquot of the extract was used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 ml/min to a Hypersil 5 μm ODS (25 cm \times 4.5 mm i.d.) column. A Waters C18 Guard Pak precolumn was used to protect

the analytical column. The Detector was a Waters 486 variable wavelength detector set at 272 nm with a Dell 486 data system and MilleniumTM software. The mobile phase consisted of acetonitrile–0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid 70:30). Quantitative analysis was achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05–5 $\mu\text{g/ml}$. The sensitivity of the assay was 0.05 $\mu\text{g/ml}$.

Serum cholinesterase. Prepared test kits (Sigma, St. Louis MO, 420-MC) were used to measure cholinesterase activity. This assay is based on the method of Rappaport et al (14), and depends on the quantitative formation of acetic acid from acetylcholine in the presence of an acid-based indicator, *m*-nitrophenol. All assays were done in triplicate.

Brain cholinesterase. Half a brain (approximately 0.9 g) was placed in a 15-ml conical polypropylene tube with 5 ml of Dulbecco's phosphate-buffered salt solution. The tissue was homogenized in a Tissue Tearor (model 985-370) for about 2 min. Tubes were then capped and centrifuged at 4000 rpm for 20 min at 4°C . The supernatant was then assayed as described above.

RESULTS

Serum samples were analyzed for the presence of PB and the extent of cholinesterase inhibition following PB administration. Acute administration of 10 mg/kg PB resulted in serum levels that averaged 175 ng/ml, \pm 32.42 ng/ml (SEM). PB could not be detected in three of the six serum samples obtained 30 min following the final administration of 1.5 mg/kg PB, but PB averaged 83 ng/ml, \pm 7.23 ng/ml (SEM) in the serum of the remaining three subjects. Acute administration of 10 mg/kg PB resulted in a 57% decrease in serum cholinesterase levels compared to vehicle administration, $t(10) = 3.11$, $p < 0.01$. Similarly, repeated administration of 1.5 mg/kg/day for 7 days decreased serum cholinesterase activity compared to vehicle administration by about 47%, $t(9) = 2.53$, $p < 0.03$. Acute or repeated PB administration did not affect brain cholinesterase levels.

Figures 1 and 2 show the cumulative number of reinforced responses on the operative lever for individual subjects during the 0-s delay condition (Fig. 1) and the 16-s delay condition (Fig. 2) after acute and repeated vehicle administration (left panels) and after acute and repeated PB administration (right panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever. Note the difference in the vertical axes between Figs. 1 and 2.

The data shown in Figs. 1 and 2 suggest that both delay duration and PB administration affected the number of responses on the operative lever. Note that some subjects failed to acquire the operant response altogether, especially following acute PB administration in the 16-s delay condition. Responses on the operative lever were analyzed by ANOVA, which included the between-subject variables delay (0 s or 16 s), treatment (acute or repeated), and drug (PB or vehicle) and the within-subject variable time (cumulative number of responses observed at each full hour of the experimental session). A number of relevant observations may be described. First of all, subjects exposed to the 0-s delay condition obtained more food pellets than those exposed to the 16-s resetting delay condition [delay: $F(1, 39) = 54.81$, $p < 0.0001$]. Secondly, all subjects obtained more food pellets as the session progressed [time: $F(7, 280) = 31.95$, $p < 0.001$]. There were no

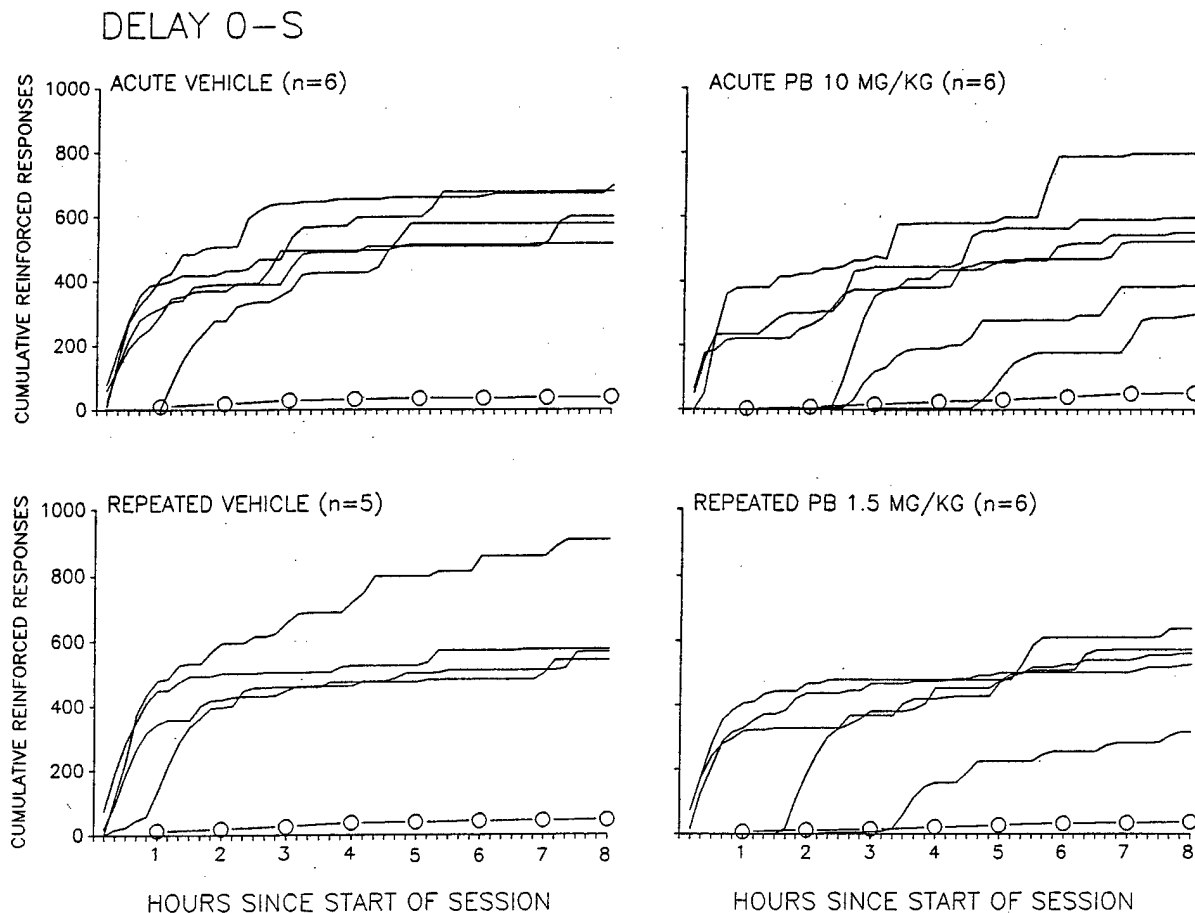


FIG. 1. The cumulative number of reinforced responses for individual subjects during the 0-s delay condition after acute or repeated vehicle administration (left-hand panels) and following the acute administration of 10 mg/kg PB or the repeated administration of 1.5 mg/kg PB for 7 consecutive days (right-hand panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever.

differences between groups as a function of acute or repeated drug administration [treatment: $F(1, 39) = 0.11$, NS]. PB did not appear to affect the number of obtained food pellets [drug: $F(1, 39) = 2.85$, $p < 0.099$], but a significant three-way interaction involving the variables delay, drug, and time, $F(7, 280) = 2.98$, $p < 0.0050$, suggested drug involvement. It can be seen in Figs. 1 and 2 that 1) subjects were more likely to earn food pellets during the earlier parts of the session during the 0-s delay condition than during the 16-s delay condition [delay \times time: $F(7, 280) = 10.24$, $p < 0.0001$]; 2) that PB administration delayed the onset of responding in some, but not all of the subjects in the drug-treated groups [drug \times time: $F(7, 280) = 1.88$, $p < 0.0734$, NS]; and 3) that the 0-s and 16-s delay conditions did not differentially affect the number of obtained food pellets following vehicle or PB administration [delay \times drug: $F(1, 39) = 0.06$ NS]. ANOVA of the latencies until the first, fifth, and tenth reinforced response during the different experimental conditions (see Figs. 1 and 2) revealed a significant interaction between delay duration and drug treatment [delay \times drug: $F(1, 39) = 4.21$, $p < 0.0468$], suggesting that latencies were longer in the 16-s delay condition [delay: $F(1, 39) = 3.48$, $p < 0.0696$] and following PB administration [drug: $F(1, 39) = 4.00$, $p < 0.0524$].

Figures 1 and 2 also reveal that the number of responses on the inoperative lever varied as a function of experimental conditions. ANOVA revealed that the number of responses on the inoperative lever was higher during the 16-s delay condition than during the 0-s delay condition [delay: $F(1, 39) = 7.57$, $p < 0.0090$] and that their number increased over time [time: $F(7, 280) = 17.98$, $p < 0.0001$], but more so during the 16-s delay condition than during the 0-s delay condition [delay \times time: $F(7, 280) = 4.13$, $p < 0.0002$]. Many more inoperative responses were observed during vehicle than during PB administration [drug: $F(1, 39) = 4.76$, $p < 0.0352$], attributable mostly to a much higher number of responses on the inoperative lever during the acute administration of vehicle in the 16-s delay condition than in any of the other experimental conditions [delay \times drug: $F(1, 39) = 3.94$, $p < 0.0541$, and treatment \times drug: $F(1, 39) = 6.62$, $p < 0.0140$].

DISCUSSION

The results of this experiment confirm and extend observations from other studies. Experimentally naïve rats exposed to 0-s delay condition obtained many more food pellets than rats exposed to the 16-s resetting delay condition. As such, these

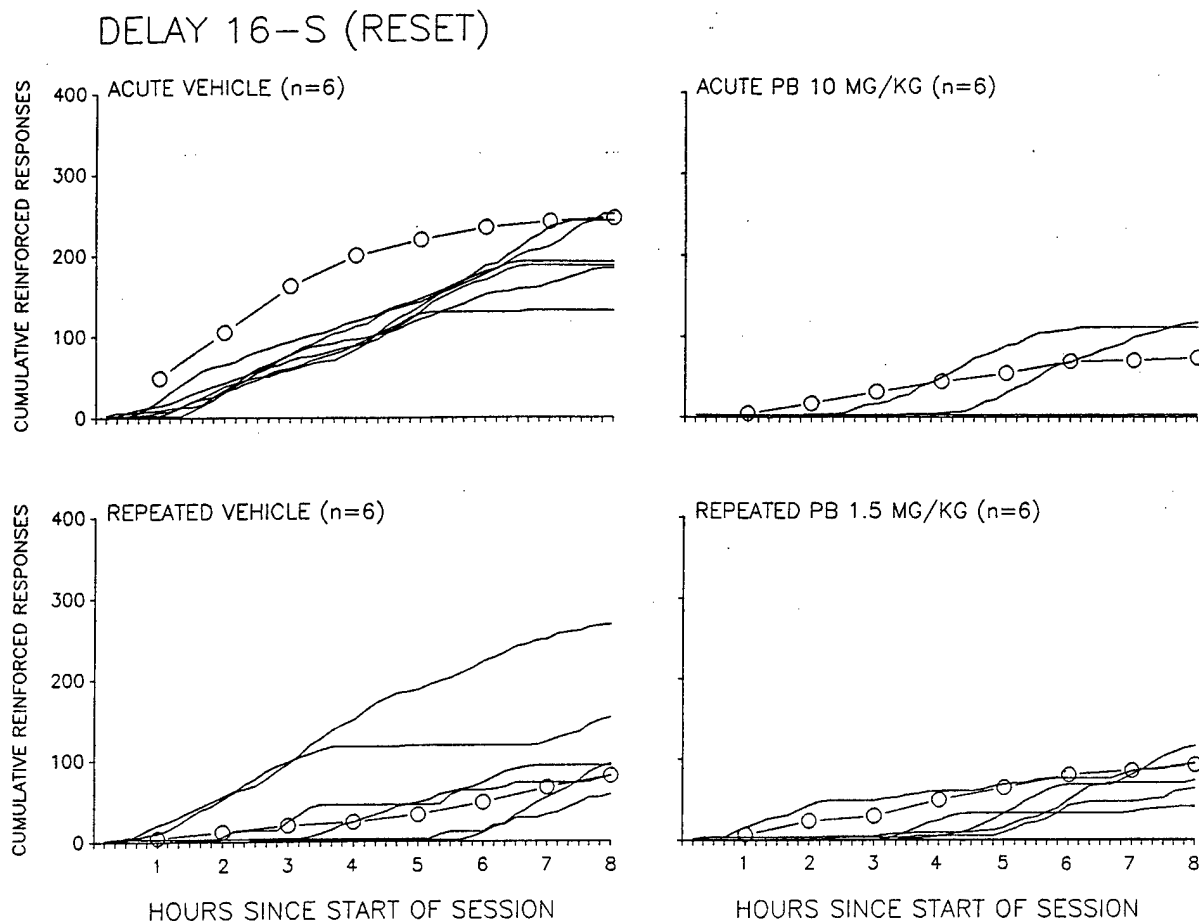


FIG. 2. The cumulative number of reinforced responses for individual subjects during the 16-s, resetting delay condition after acute or repeated vehicle administration (left-hand panels) and following the acute administration of 10 mg/kg PB or the repeated administration of 1.5 mg/kg PB for 7 consecutive days (right-hand panels). The open circles connected by the solid lines represent group-averaged cumulative responses on the inoperative lever.

results confirm those of other experiments in which it was shown that response-contingent delayed pellet presentation delays response acquisition (11,19). Acute administration of 10 mg/kg PB and repeated administration of 1.5 mg/kg/day for 7 days reduced cholinesterase activity by approximately 50%. There were no differences between groups as a function of acute or repeated PB administration, indicating that the cumulative effects of very small doses of PB (1.5 mg/kg/7 days) were similar to those of one much larger dose of PB (10 mg/kg). The data also showed that PB administration delayed the onset of responding in some, but not all, subjects in the PB-treated groups. These data imply that repeated administration of a very small dose of PB (1.5 mg/kg/day for 7 days) adversely affects response acquisition in experimentally naive subjects. It should be noted that the repeated dose of PB was chosen to resemble that which was most commonly administered during the Gulf War, although Gulf War exposure may have been more prolonged (i.e., 3×30 mg/70 kg for 7–14 days). That particular treatment regimen has been stated to be safe and well tolerated in a double-blind evaluation of its safety, tolerance, pharmacokinetics, and pharmacodynamics in 90 male and female volunteers (10). These pharmacokinetic studies, however, did not assess any functional conse-

quences of such drug administration regimen. The results of the present experiment appear to indicate that the functional consequences of this low dose of PB (lower than those that have been reported to facilitate drug interactions with such compounds as permethrin and DEET [cf. (1,2)] should not be underestimated.

An interesting question is whether PB causes these effects on behavior by acting on the central nervous system (CNS) or on the peripheral nervous system (PNS). It has been assumed that PB, as a quaternary carbamate, does not cross the blood-brain barrier (BBB). If that is true, it would seem that PB's behavioral effects should result from actions only on the PNS. However, there are a number of findings that indicate that PB's effects may be centrally mediated. First, PB at low doses that do not cause signs of toxicity, produced behavioral effects in paradigms that involve CNS activity (21). Secondly, pretreatment with PB protects against intoxication with soman, an OP nerve gas that predominantly acts in the CNS (3). Furthermore, disruption of the BBB might possibly allow PB administration to have central effects. Friedman et al. (4) showed in stressed mice that an increase in BBB permeability reduced the dose of PB required to inhibit brain AChE activity by 50% to less than $1/100$ th of the dose required in non-

stressed mice. When PB was given to healthy volunteers during peacetime, only 8.3% of the subjects reported CNS symptoms (headaches, insomnia, drowsiness, nervousness, unfocused attention and impaired calculation capacities), whereas in soldiers treated during the Gulf War, 23.6% reported CNS symptoms, possibly due to enhanced stress levels under those conditions (4,6).

Although PB appears to have central effects, Liu (13) has argued that the detrimental effects of PB on operant behavior are mediated by peripheral muscarinic receptors. Liu studied the effects of atropine, a muscarinic antagonist with both a central and a peripheral action, and methylatropine, a muscarinic antagonist with only a peripheral action, on PB-induced (12 mg/kg) behavioral disruption during a brightness discrimination task. Atropine partially antagonized the PB-induced reinforcement loss, while at the same time increasing the number of nonreinforced responses. However, methylatropine completely antagonized the PB-induced reinforcement loss as well, without affecting the number of nonreinforced responses. This suggests that the detrimental effects of PB on operant behavior are due to the stimulation of peripheral muscarinic receptors, possibly in the gastrointestinal tract, because in humans, gastrointestinal disturbances are a common side effect of PB administration (18). Other studies conducted in our laboratories (8) have shown that acute PB administration at 10 mg/kg results in a sex-dependent decrease in locomotor activity in male and female Sprague-Dawley rats. This observation suggests that the effects of PB administration, at least in the acute conditions, may have produced effects on

motor behavior that could have interfered with response acquisition as studied in the present experiment or the decrease may be symptomatic of the general malaise caused by PB. There are currently no data available with respect to the locomotor effects of repeated administration of very small doses of PB. The present experiment was not designed to evaluate these alternative explanations, but such experiments should be conducted in the future to arrive at a comprehensive understanding of the effects of acute and repeated PB administration on response acquisition. In particular, it might be worthwhile to determine PB effects on response acquisition in rats pretreated with methylatropine or methylscopolamine to block peripheral cholinergic muscarinic receptors.

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THE EFFECTS OF PYRIDOSTIGMINE BROMIDE AND PERMETHRIN, ALONE OR IN
COMBINATION, ON RESPONSE ACQUISITION IN MALE AND FEMALE RATS

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It has been hypothesized that concurrent exposure to pyridostigmine bromide and permethrin may have contributed to the development of neurocognitive symptoms in Gulf War veterans. The present experiment was designed to investigate the effects of pyridostigmine bromide and permethrin alone, or in combination, on the acquisition of a novel response, one measure of normal cognitive functioning. Male and female Sprague-Dawley rats were treated with pyridostigmine bromide (1.5 mg/kg/day, by gavage in a volume of 5 ml) or its vehicle for seven consecutive days. They then also received an intraperitoneal injection of permethrin (0, 15 or 60 mg/kg) before they were exposed to an experimental session during which they could earn food by pressing a lever in an operant chamber. Serum permethrin levels increased as a function of its dose and were higher in rats treated with pyridostigmine bromide. Sex differences were observed as permethrin levels were higher in female rats than in male rats following the highest dose. Pyridostigmine bromide delayed response acquisition in male and female rats, and resulted in higher response rates on the inactive lever in female rats than in male rats. Although permethrin levels were higher in subjects treated with pyridostigmine bromide than in those treated with vehicle, there were no differences in the behavioral effects of permethrin. Whether or not these behavioral effects of pyridostigmine bromide are of central or peripheral origin will need to be determined in future studies as its effects on motor activity and/or gastro-intestinal motility may have affected response acquisition.

Key words: Gulf War Illness, pyridostigmine bromide, permethrin, cholinesterase inhibition, synergism, learning, response acquisition, lever press, male and female rats

Concurrent exposure to pyridostigmine bromide (PB), a carbamate cholinesterase inhibitor, and the pyrethroid insecticide permethrin (PERM) may have contributed to the development of a syndrome that appears to have afflicted military personnel who served during the Gulf War (5, 9, 10, 11, 14, 26).

PB is a quaternary ammonium compound that inhibits the hydrolysis of acetylcholine (ACh) by competitive reversible binding to acetylcholinesterase (AChE). It has been suggested that PB may decrease nerve gas toxicity by occupying AChE binding sites (33). Reportedly, PB was taken prophylactically during the Gulf War (three x 30 mg / day / 70 kg for up to 21 days) when there was a high risk of nerve gas exposure (14).

The synthetic pyrethroids, of which PERM is one, are widely used insecticides that have been divided into two classes according to their chemical properties and toxicity symptoms (32). Toxic exposure to PERM, a Type I compound, is evidenced by aggressive sparring, hypersensitivity to external stimuli, whole body tremor and prostration in experimental animals (cf.19). These symptoms are thought to originate in the central nervous system as they have been shown to correlate with the concentration of unmetabolized pyrethroid in brain tissue (8). PERM was used to impregnate battle-dress uniforms in the field during the Gulf War, but the extent of its usage is not known.

Some of the behavioral effects of small doses of PB and PERM have been documented before. Wolthuis and Vanwersch (33) determined in rats that PB decreased two-way shuttle-box avoidance efficiency, decreased open-field locomotor activity and produced a dose-dependent decrease in the number of correct steps in a hurdle-stepping task, at less than 10% of the intraperitoneal LD₅₀. In other studies, Liu and his colleagues (16,17,22) tested the effects of PB on schedule-controlled behavior. They observed that low doses of PB (3-12 mg /kg, by gavage) which did not produce any overt signs of toxicity, decreased fixed-ratio (FR) 30 response rates, whereas higher doses (30 and 40 mg/kg) completely eliminated responding. It has recently been reported that PB dose-dependently decreased locomotor activity in male and female rats, but that

female rats were affected by lower doses than males (13). In another experiment we showed that acute and repeated PB administration delayed response acquisition when reinforcers were either presented immediately after a response or following a short delay (30).

Small doses of PERM which did not produce any overt signs of neurotoxicity have been shown to dose-dependently decrease responding maintained by a variable-interval 20-s (VI 20-s) schedule of reinforcement (3). When rats were trained to respond on a variable-ratio 25 (VR 25) schedule (23), the highest dose of PERM (60 mg/kg, IP) significantly decreased response rates. Peele and Crofton (21) exposed male Long-Evans hooded rats to a four-component multiple (VI 10-s, VI 30-s, VI 90-s, VI 270-s) schedule of food reinforcement and tested different doses of PERM (vehicle, 100, 200, 300 and 400 mg/kg) which they administered *per os*, 90 min prior to the start of the session. Response rates decreased dose-dependently and the oral ED₅₀ was established at 350 mg/kg in this experiment.

It has been reported that the neurotoxicological effects of PB and PERM combinations may exceed the effects of the individual compounds. McCain, Lee, Johnson, Whaley, Ferguson, Beall and Leach (18) assessed the LD₅₀ of PB and PERM either alone, or in combination, and reported that different doses of PB in combination with PERM killed more male laboratory rats than would have been expected if the effects of the compounds had merely been additive. Similarly, Abou-Donia and his colleagues have recently shown in hens that the behavioral and neurotoxicological effects of combined treatment with PB and PERM exceeded those observed after administration of the individual compounds (1). These investigators suggested that the effects of the compound combinations might be a function of the fact that PERM is more likely to penetrate the central nervous system when PB is present in the circulation.

It has been suggested that the intellectual and neurocognitive functioning in veterans presenting Gulf War Syndrome may have been compromised by concurrent exposure to PB and PERM, or other compounds employed in the war theatre (12). The present experiment is one in a series of studies designed to assess the effects of small, but behaviorally active doses of PB and

PERM, alone or in combination, on different behavioral endpoints, in this case the acquisition of a novel response (learning). In these experiments, naive, food-restricted, subjects are given the opportunity to obtain food by pressing one of two levers in an experimental chamber. Previous experiments have shown that untreated control subjects quickly learn to press the lever associated with reinforcement presentation (15, 24, 25, 27), but that acute and repeated PB administration delayed response acquisition (30). It was hypothesized that concurrent PB and PERM administration might further delay the acquisition of a novel response.

Different groups of male and female subjects were treated with an amount of PB approximately equal to the Gulf War dose (1.5 mg /kg /day for seven days by gavage), or they were treated with distilled water. They then received an intraperitoneal injection of PERM (vehicle, 15 or 60 mg/kg) before an experimental session during which lever presses (novel response) were followed by food presentation. The doses of PERM were chosen to reflect those that had been behaviorally active in other experiments. Whether or not these doses approximate potential Gulf War exposure levels has not yet been determined. The lever press response was not shaped in any way, but left to emerge spontaneously. Male and female rats participated in this experiment because it has been shown that the behavioral consequences of PB and PERM administration, just like those of other substances, may be affected by sex hormones (2, 13, 20, 28, 29, 31).

METHODS

Subjects. Forty-eight male and 48 female Sprague-Dawley rats were obtained from a commercial supplier (Zivic-Miller, Zelienople, PA) when they weighed approximately 225 - 250 g. They were housed in same-sex pairs under a reversed light-dark cycle (lights on 6:00 p.m.) and allowed free food and water for one week. Access to food was then limited for the remainder of the

experiment (16 g/day per male rat and 12 g/day per female rat, offered at 5:00 p.m.), while tap water remained continuously available.

Apparatus. The experiment was conducted in six identical Coulbourn Instruments modular rodent operant-conditioning chambers, which were 25 cm wide, 30 cm long and 29 cm high (Allentown, PA). The sidewalls of each chamber were made of Plexiglas; the back wall and the intelligence panel were made of stainless steel. The floor consisted of 16 rods, spaced 2-cm apart (center to center). Two retractable rodent levers were located symmetrically to the side of the pellet tray, 6.3 cm from the floor of each chamber. When extended, the levers protruded 1.8 cm from the intelligence panel and required a force of more than 0.20 N to be operated. There were three stimulus lights directly above each lever and a house light was located 3 cm from the ceiling in the middle of the intelligence panel. The pellet tray was illuminated by a white light bulb during the delivery of a food pellet (Noyes, 45 mg purified rodent formula). Each experimental chamber was housed in an individual sound-attenuating, ventilated cabinet. The chambers were connected to an IBM-PC compatible microcomputer (GatorByte, Gainesville, FL) through a LabLinc interface (Coulbourn Instruments LPC, Allentown, PA) located in the experimental room itself. Experimental contingencies and data acquisition procedures were programmed in L2T2 (Coulbourn Instruments LPC, Allentown, PA).

Procedure.

Magazine training. The subjects were placed in the darkened operant chamber from which the levers had been retracted five minutes before the start of the session. At the beginning of the magazine training session, the house light was illuminated. Pellet delivery, which was accompanied by brief illumination of the light in the pellet tray, was then programmed to occur once every 60 s, on average, on a random-time (RT) schedule until 60 pellets had been presented. Most subjects had retrieved all pellets from the tray at the end of the session, the few subjects

who had not, received an additional training session. Magazine training was completed before any drugs were administered.

Response acquisition session. Subjects were put into the dark operant chamber five minutes before the beginning of the session at 4:00 p.m., to include the final two hours of the subjects' dark period. Experimental sessions had to be arranged in this manner so as not to interfere with other experiments that were being conducted during the regular daytime hours. The house light was illuminated at the beginning of the session and the two levers were extended into the experimental chamber. During the response acquisition session, each press on the left (operative) lever immediately resulted in the presentation of a food pellet, while a press on the right (non-operative) lever was recorded but did not have any scheduled consequences. The experiment was terminated after eight hours and the subjects were immediately removed from the experimental chamber. All response acquisition sessions were conducted on consecutive days.

Drug administration. Half of the subjects received distilled water once a day for six consecutive days at 4:00 p.m. On day seven, some subjects received distilled water 15 min prior to the administration of PERM vehicle which occurred 15 min prior to the beginning of the experimental session (n=7 male rats, n=5 female rats). Other subjects received distilled water followed by 15 mg/kg PERM (n= 8 male rats, n=4 female rats), while the remaining subjects received distilled water followed by 60 mg/kg PERM (n=8 male rats, n=8 female rats). The other half of the subjects received 1.5 mg/kg PB once a day for six consecutive days. On day seven, some subjects received 1.5 mg/kg PB 15 min prior to the administration of PERM vehicle which occurred 15 min before the start of the experimental session (n=7 male rats, n=8 female rats). Other subjects received 1.5 mg/kg PB and 15 mg/kg PERM (n=8 male rats, n=8 female rats) or 1.5 mg/kg PB and 60 mg/kg PERM (n=7 male rats, n=6 female rats). PB and its vehicle were administered by gavage in a volume of five ml/kg, PERM and its vehicle were administered IP in a volume of two ml/kg. All experimental groups had been designed to consist of eight subjects each, but data from some of the subjects had to be excluded from the final analyses due to

equipment malfunction during the course of some experimental sessions. The day following the response acquisition session all subjects received the same drug treatment that they had also received the day before. This allowed us to evaluate PB and PERM serum levels following a pretreatment time identical to that of the behavioral experiments. Vaginal smears were obtained from female rats before both the operant acquisition session and the next day. These samples were collected to allow us to analyze behavioral and physiological variables in the context of the stage of estrus cycle at the time of testing.

Drug preparation.

Pyridostigmine bromide (PB) was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in distilled water. Technical grade permethrin (PERM [3-phenoxyphenyl) methyl(+)-cis,trans - 3 -(2,2-dichloroethenyl) - 2,2 -dimethylchloro-propanecarboxylate], minimum 35% (+/- cis and maximum 65% (+/-) trans) was obtained from Coulston Products (Easton, PA, procured via Dr. W. McCain, Aberdeen Proving Grounds, MD) and prepared in a vehicle of equal volumes of Emulphor and 95% ethanol (total volume of 0.2 ml /10 mg of PERM). This mixture was diluted with 0.9% physiological saline to the desired concentrations.

Serum preparation.

Each rat was placed in a jar containing a paper towel saturated with Metofane (Methoxyflurane) for less than one minute. The anesthetized animal was then quickly decapitated. Blood was collected in a 15-ml polystyrene culture test tube and allowed to coagulate on ice for two hours. It was then centrifuged for 15-20 minutes at approximately 3000 revolutions per minute. The serum was drawn off the solid cell matter with a clean glass Pasteur pipette and placed in a 1.5 ml polystyrene microcentrifuge tube. It was then immediately placed in a freezer (at -20 degrees Centigrade) where it was stored until analysis.

Neurochemical analyses

Pyridostigmine bromide. The 0.5-ml serum sample was transferred to a stoppered tube and vortexed with 1ml of 0.025M potassium phosphate buffer at pH 3. This mixture was then applied to a Strong Cation Exchange column which had previously been conditioned under vacuum on a Vac Elut manifold (Analytichem) with methanol (2 mL), water (1 mL) and 0.25M phosphate buffer (1mL). After application of the sample, the column was air dried for approximately 30 seconds and then washed with phosphate buffer (1 mL) and 0.1M acetic acid. The column was again air dried for 30 seconds before eluting off the adsorbed drugs with ammoniacal methanol (3%, 2 mL). The final extract was evaporated to dryness under nitrogen and the residue reconstituted in 50 μ l of methanol. A 20 μ l aliquot of the extract was used for HPLC analysis. This analysis was performed using a Waters 510 pump to deliver solvent at 1 mL/min to a Hypersil 5 μ m ODS column (25cm x 4.5mm ID) column. A Waters C18 Guard Pak precolumn was used to protect the analytical column. The Detector was a Waters 486 variable wavelength detector set at 272nm with a Dell 486 data system and Millenium (TM) software. The mobile phase consisted of acetonitrile-0.1% triethylamine in water (adjusted to pH 3.2 with phosphoric acid 70:30). Quantitative analysis was achieved by comparison of peak areas with unextracted standards. Each determination was taken as the mean of three replicate injections. The calibration graph was produced over the range of 0.05-5 μ g/ml.

Permethrin. A 200-mg Clean Screen solid phase extraction cartridge (sorbent type CSDAU, manufactured by Worldwide Monitoring) was conditioned with 2 mL acetone, 2 mL methanol and 2 mL deionized water. A 0.5 mL volume of sample rat serum was transferred to the cartridge reservoir and allowed to percolate by gravity through the sorbent bed. The cartridge was washed with 2 mL deionized water, placed on a vacuum manifold and dried under full vacuum for approximately 5 min. Permethrin was eluted from the cartridge with a 1mL volume of acetone and collected in a graduated conical tube. A 10 μ L volume of internal standard (40

ng/ μ L of US108, purchased from Ultra Scientific) was added to the tube, the final volume was adjusted to 1 mL and the extract was transferred to a gas chromatograph vial for analysis.

The extracts were analyzed for permethrin using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass selective detector operating in the electron impact mode. The gas chromatograph was equipped with a 30m HP-5MS column (250 μ m diameter with a 0.25 μ m film thickness) operated in the splitless mode at a flow rate of 0.8 mL/min. A 1 μ L aliquot of the final extract was injected into the gas chromatograph and the analytes were separated using the following temperature program. The inlet temperature was set at 275 degrees C and the initial oven temperature was set at 40 degrees C. The initial oven temperature was held for 4 min and then ramped to 270 degrees C at 10 degrees C/min. The oven was held at 270 degrees C for 5 min then ramped to 300 degrees C at 25 degrees C/min. the final temperature was maintained for 6.8 min. Under these conditions, the retention times for cis-permethrin and trans-permethrin were 27.6 min and 27.8 min respectively. The detector was operated in the selected-ion-monitoring mode with an electron impact voltage of 70eV and an electron multiplier voltage of 1882 V. Both forms of permethrin were quantitated using ion 183 and confirmed using ions 163 and 165. The internal standard was quantitated using ion 188.

Statistical Analyses

The experimental design included SEX (male and female rats), two levels of repeated drug administration (PB or vehicle), three levels of acute pesticide administration (PERM vehicle, 15 mg/kg or 60 mg/kg) and TIME (repeated observations within the experimental session). PB levels and PERM levels were subjected to a three-way analysis of variance to analyze the effects of the subjects' gender (SEX), chronic PB administration (PB) and acute PERM administration (PERM). The number of responses at each hour of the experimental session was analyzed by analysis of variance which included these same three factors and the factor TIME (repeated within subjects). Repeated measures analysis of variance allowed us to assess the effects of the subjects'

gender, chronic PB administration, acute PERM administration and TIME since the start of the session on response acquisition.

RESULTS

Figure 1 shows serum PERM levels for male and female rats treated with different doses of PERM in the presence or absence of PB. Serum levels of PERM increased as a function of PERM dose ($F(1,43) = 52.68, p < 0.0001$) and were higher in rats treated with PB than in rats treated with distilled water only ($F(1,43) = 4.79, p < 0.03$). There was a significant three-way interaction between SEX, PB and PERM ($F(1, 43) = 21.17, p < 0.0001$). Post-hoc analyses showed that serum PERM levels were higher in females than in males following 60 mg/kg PERM both in PB-treated and vehicle-treated subjects ($p < 0.0027$ and $p < 0.0001$, respectively). It should be noted that repeated administration of 1.5 mg/kg PB resulted in PB serum levels 30 min following the final administration of PB that were below the detection limit of our assay in all groups of subjects.

 Insert Figure 1 about here

The behavioral results of the experiment are presented in Figures 2, 3 and 4. They show the cumulative number of reinforced responses on the active lever observed in 10 min segments of the experimental session for individual male (Figure 2) and female subjects (Figure 3). The data for subjects treated with PB vehicle and the different doses of PERM are shown in the left-hand panels of Figures 2 and 3, those for subjects repeatedly treated with 1.5 mg/kg PB and the different doses of PERM are shown in the right-hand panels. Open circles reflect the number of responses observed on the inactive lever during every hour of the experimental session.

Insert Figures 2 and 3 about here

Figure 4 presents these same data, but averaged over groups of subjects to allow for more direct comparisons of the data across drug conditions and gender. The data for male and female rats are shown in the left- and right-hand panels respectively, the top panels show the effects of PERM in vehicle-treated subjects, the bottom panels those in PB-treated subjects. The filled symbols show cumulative lever presses in subjects treated with PB vehicle and PERM vehicle, i.e. essentially untreated subjects.

Insert Figure 4 about here

Control male and female rats treated with PB and PERM vehicles (upper left-hand panels in Figures 2 and 3) quickly and consistently initiated responding on the operative lever from the onset of the experimental session. PB administration reduced the number of responses on the operative lever as a function of the time since the beginning of the session (PB x TIME: $F(7,518) = 2.56$, $p < 0.0133$; TIME: $F(7,518) = 38.90$, $p < 0.0001$; PB: $F(1,72) = 3.52$, $p < 0.0648$) in male and female rats. Post-hoc analyses showed a higher number of reinforced responses when subjects had been treated with distilled water than when they had been treated with PB during the first two hours of the experimental session. PERM administration did not affect response acquisition. Sex differences were not observed in response acquisition on the active lever, but PB administration affected overall response rates (responses per minute) in the inactive lever in a sex-dependent manner (SEX x PB: $F(1,72) = 5.50$, $p < 0.0217$). There was no difference between males and females following the administration of PB vehicle, but females responded much more on the inactive lever than males following repeated PB administration ($p < 0.0096$). Analysis of

vaginal smears did not reveal any obvious relationship between stage of estrus cycle at the time of testing and any of the behavioral and physiological measures.

DISCUSSION

The present experiment was designed to investigate to what extent repeated PB administration alone, or in combination with the acute administration of PERM would affect response acquisition in male and female rats. PB's repeated dose was chosen to mimic that of possible, short-term, Gulf War exposure (1.5 mg/kg PB by gavage in 5 ml/kg). The doses of PERM were similar to those previously shown to be behaviorally active, yet not toxic (e.g. 23).

The experiment has yielded a number of interesting observations. Serum PERM levels increased as a function of its dose and, in the presence of repeated PB administration, they were higher in female rats than in male rats following the highest dose of PERM (60 mg/kg). These observations are interesting in the context of observations by others (1) that the behavioral and neurotoxicological effects of the combined treatment with PB and PERM exceeded those observed after the administration of the individual compounds. The results of the present experiment suggest that even very low, but repeatedly administered, doses of PB may not only affect PERM serum levels, but that they do so in a sex-dependent manner. These sex differences in the neurochemical interactions between PB and PERM warrant further scrutiny in future experiments. This especially in view of the fact that others have reported that PB actually may reduce PERM penetration into the brain (4). Analysis of vaginal smears obtained in this experiment in the context of PERM levels did not reveal any obvious correlations but such should not be taken to indicate that repeated PB administration is without gender-dependent effects (vide supra). Experiments in intact and gonadectomized male and female rats with and without hormone replacement (testosterone propionate, estradiol and progesterone) should shed more light on these questions.

PB administration delayed the time at which responding was initiated and decreased the number of reinforced responses in male and female rats. As such these results confirm and extend those of another study in which it was shown that acute and repeated PB administration lowered the number of reinforced responses when response acquisition was examined under conditions of immediate and delayed reinforcement (30). PB administration resulted in higher response rates on the inactive lever in female rats than in male rats. PERM alone did not affect response acquisition in male and female rats. This observation is in contrast to those of other experiments in which it was shown that similar doses of PERM dose-dependently decreased well-established schedule-controlled performance (3, 21, 23). It is interesting to note that there were no sex differences in response acquisition despite the fact that PB administration affected PERM levels more in female rats than in male rats. This is not to say that the sex differences in neurochemical parameters do not appear to have behavioral consequences. Other studies conducted in our laboratory, for instance, have shown that acute PB administration results in a sex-dependent decrease in locomotor activity (13). It should be noted that the repeated dose of PB was chosen to resemble that which may have been used frequently during the Gulf War. The results of the present experiment in conjunction with those of other studies (13, 30) appear to indicate that the functional consequences of this low dose of PB should not be underestimated.

Did PB act on the central nervous system (CNS) or the peripheral nervous system (PNS)? It has been assumed that PB, as a quaternary carbamate, does not cross the blood-brain barrier (BBB), but a number of recent findings suggest that PB's effects may be centrally mediated. First, evidence has been presented to show that stress may make it easier for PB to penetrate the BBB. Friedman, Kaufer, Shemer, Hendler, Soreq and Tur-Kaspa (7) have shown that swim stress reduced the dose of PB required to inhibit brain AchE activity by 50% to less than 1/100th of the dose in subjects that had not been stressed. It has also been shown that PB pretreatment protects against intoxication with soman, a nerve agent that predominantly acts on the CNS (6). Finally, it appears that PB disrupts behavioral tasks that involve appropriate CNS activity (this experiment,

30, 33). However, it can not be excluded that behavioral performance may have been disrupted because of PB's effects on other systems intricately involved with learning and memory such as those which mediate motivation and motor activity.

In summary, the results of the present experiment show that repeated PB administration disrupts response acquisition in male and female Sprague-Dawley rats. They also indicate that such disruption is not observed after PERM administration and that there is not a significant behavioral interaction when PB and PERM are simultaneously administered. However, PB differentially affects serum PERM levels in male and female rats. Even though these sex-dependent neurochemical effects did not appear to have significant behavioral consequences under the present experimental conditions, it would be appropriate to further evaluate the differential contribution of gonadal hormones to the behavioral and neurochemical effects of PB and PERM in future experiments.

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FOOTNOTE

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FIGURE LEGENDS

Figure 1: Average serum permethrin levels (\pm 1 S.E.M.) in male rats (left panel) and female rats (right panel) treated with different doses of permethrin (0, 15 or 60 mg/kg) in the presence or absence of repeated administration of 1.5 mg/kg pyridostigmine bromide.

Figure 2: The cumulative number of reinforced responses observed in 10 min segments of the experimental session for individual male rats. The data for subjects treated with pyridostigmine bromide vehicle and the different doses of permethrin are shown in the left hand panels of the figure, those for subjects repeatedly treated with 1.5 mg/kg pyridostigmine bromide and the different doses of permethrin are shown in the right hand panels of the figure. Open circles reflect the number of responses observed on the inactive lever during every hour of the experimental session.

Figure 3: The cumulative number of reinforced responses observed in 10 min segments of the experimental session for individual female rats. The data for subjects treated with pyridostigmine bromide vehicle and the different doses of permethrin are shown in the left hand panels of the figure, those for subjects repeatedly treated with 1.5 mg/kg pyridostigmine bromide and the different doses of permethrin are shown in the right hand panels of the figure. Open circles reflect the number of responses observed on the inactive lever during every hour of the experimental session.

Figure 4: The average cumulative number of reinforced responses (\pm 1 S.E.M.) in the different treatment groups at each hour of the experimental session. The data for male and female rats are shown in the left- and right-hand panels respectively, the top panels show the effects of permethrin in vehicle-treated subjects, the bottom panels those in subjects treated with

pyridostigmine bromide. Circles represent data from those subjects treated with the permethrin vehicle, while triangles and diamonds represent data from subjects treated with 15 and 60 mg/kg permethrin, respectively. The filled circles show cumulative lever presses in subjects treated with the pyridostigmine bromide vehicle and the permethrin vehicle, i.e. essentially untreated subjects.

FIGURE 1

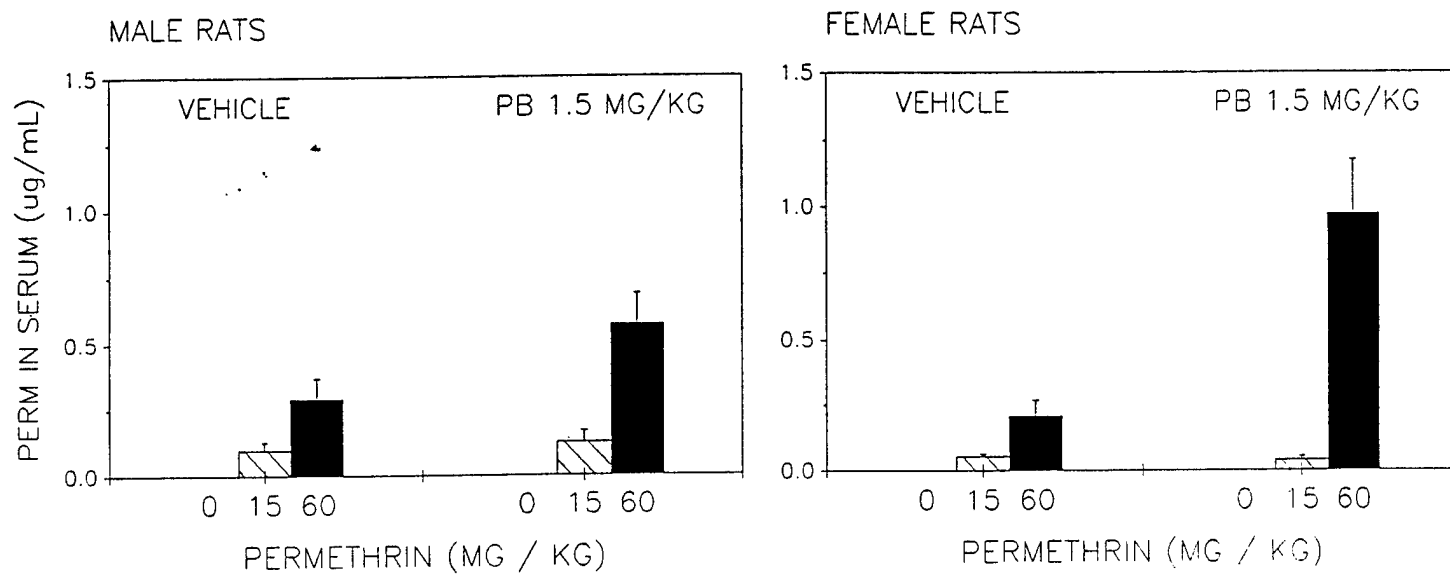


FIGURE 2

MALE RATS

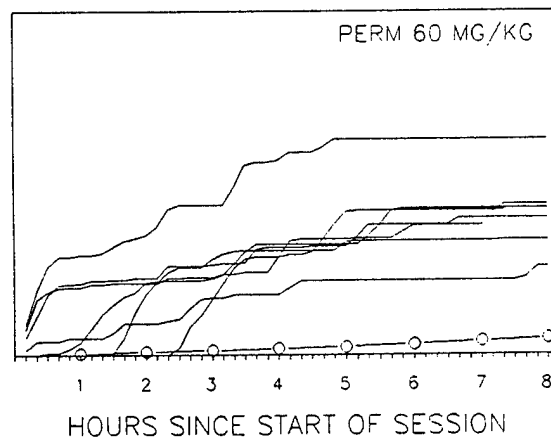
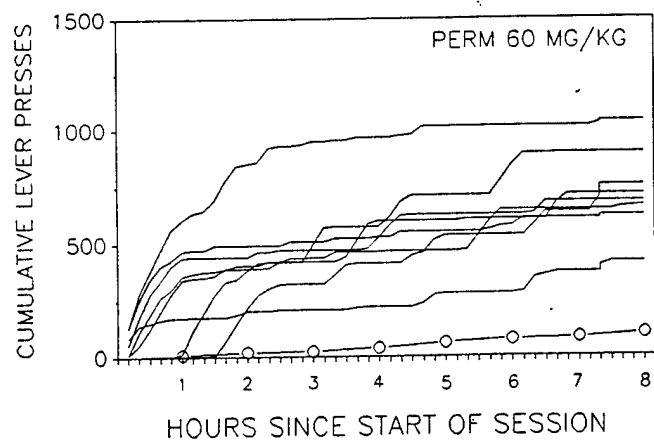
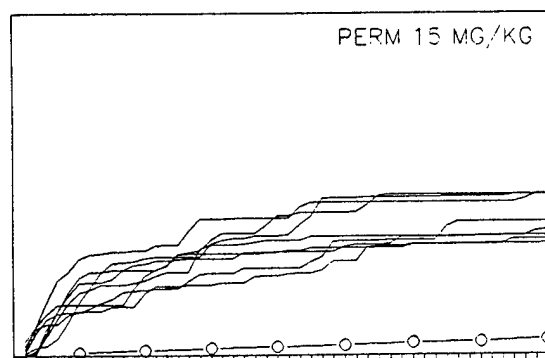
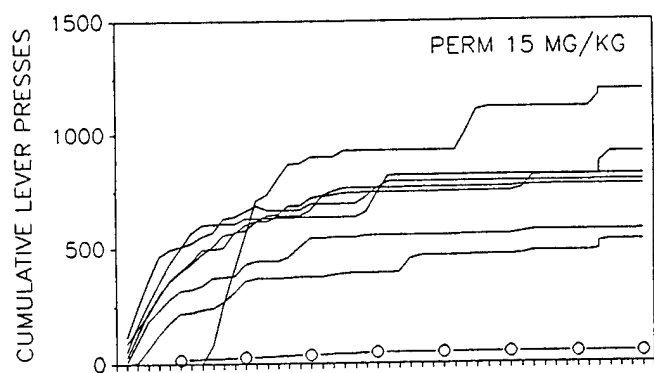
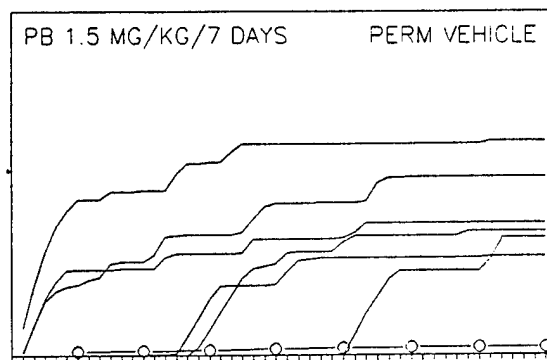
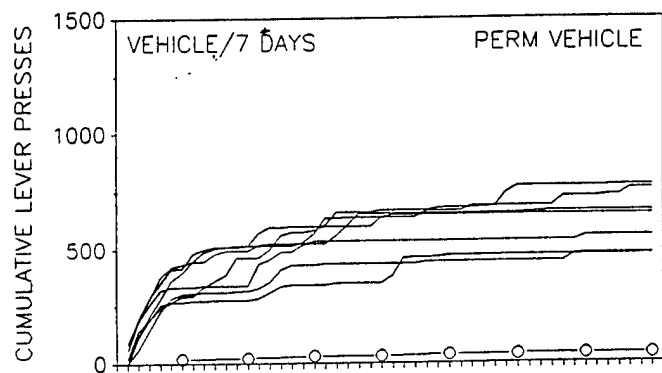


FIGURE 3

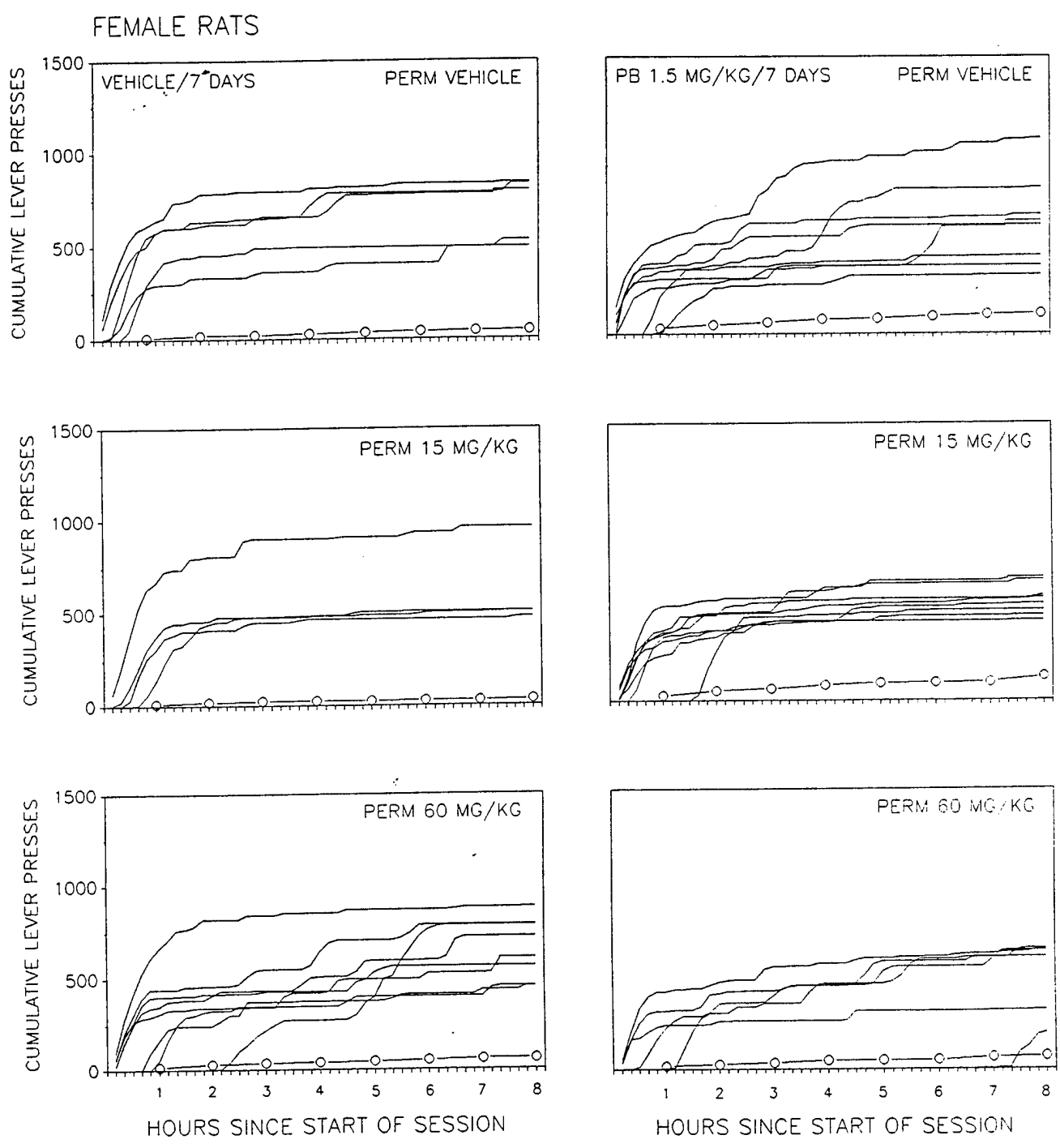


FIGURE 4

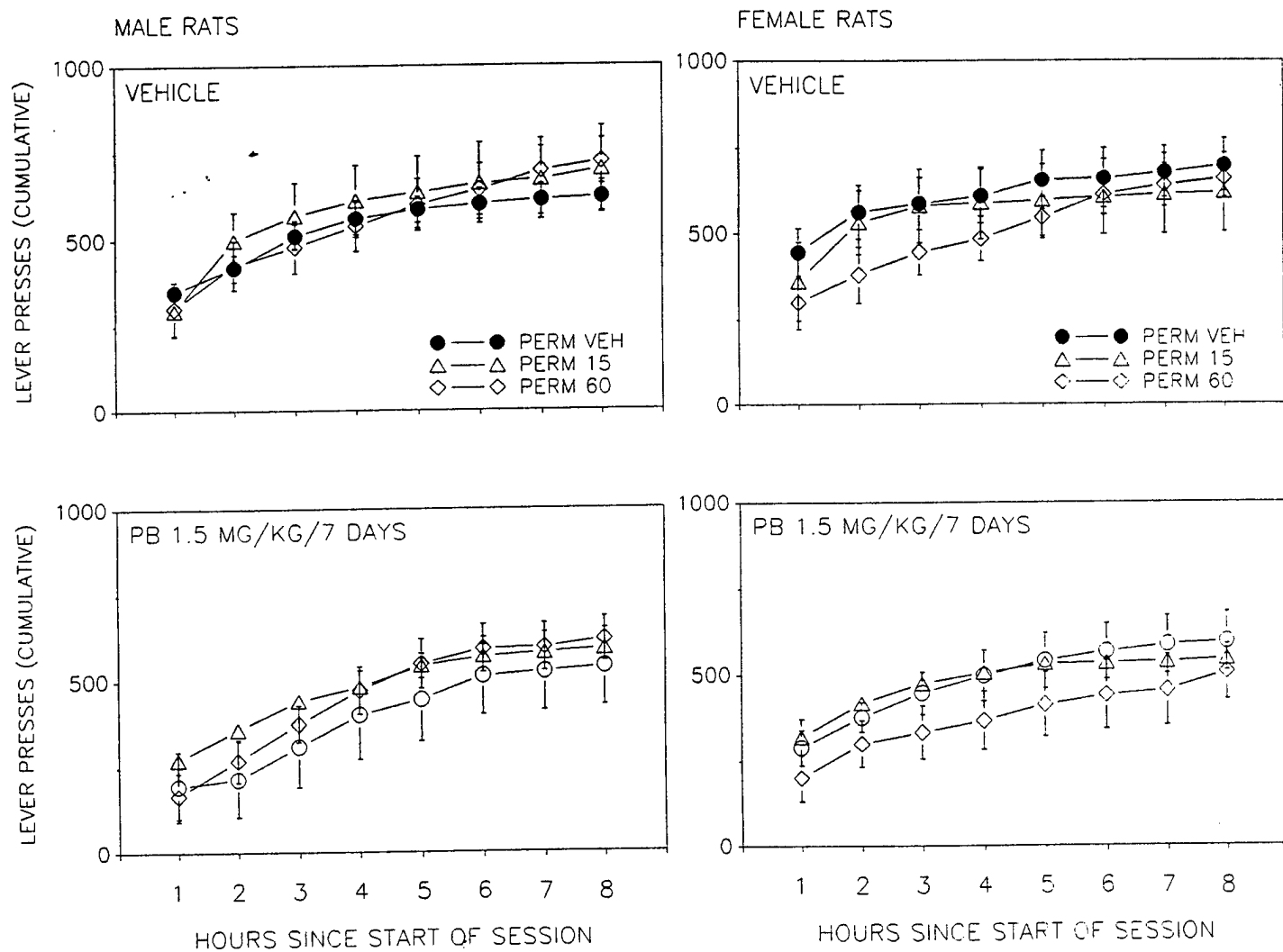


Figure 1 and Figure 2

Table 1

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FIGURE 1

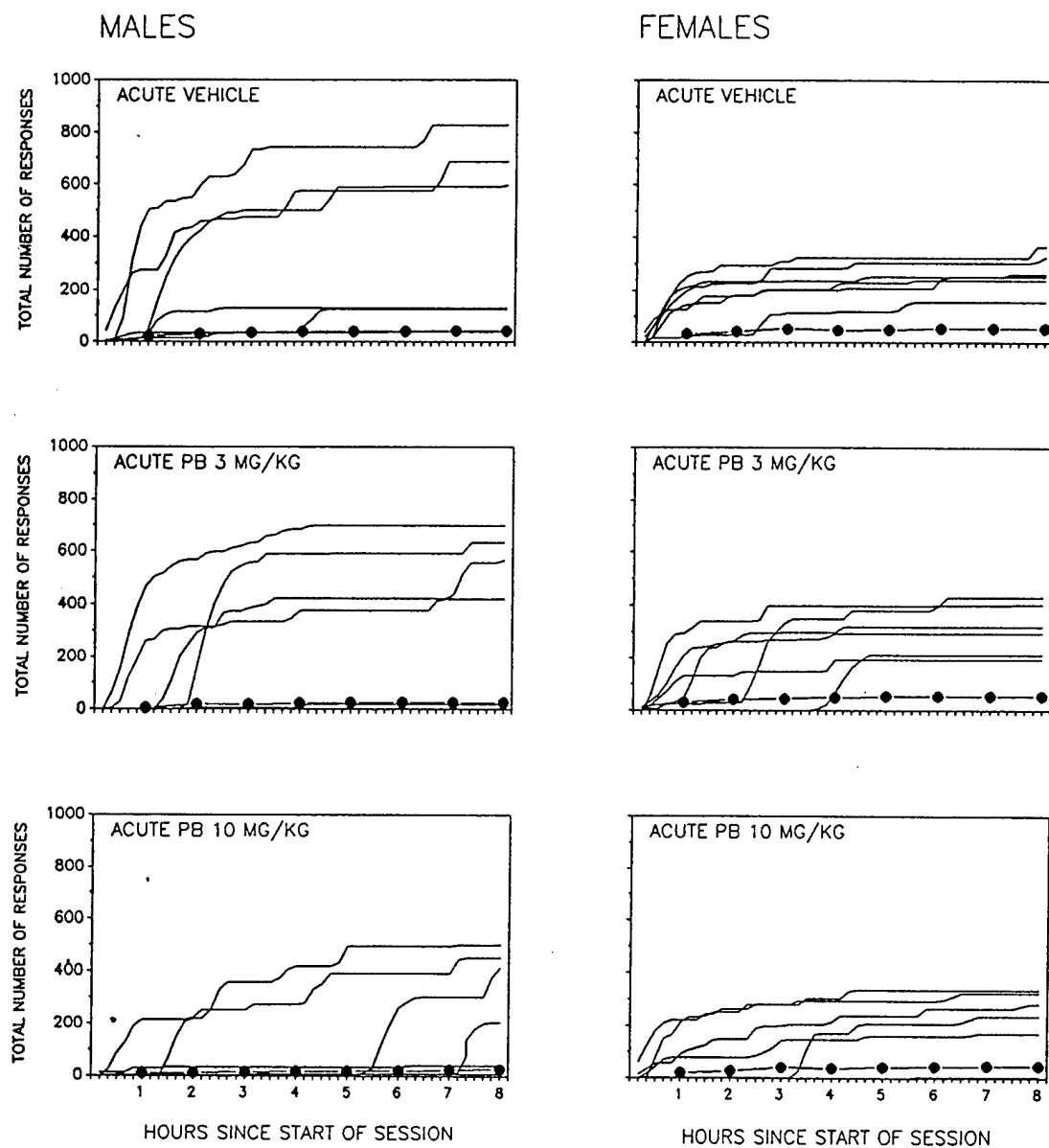


FIGURE 2

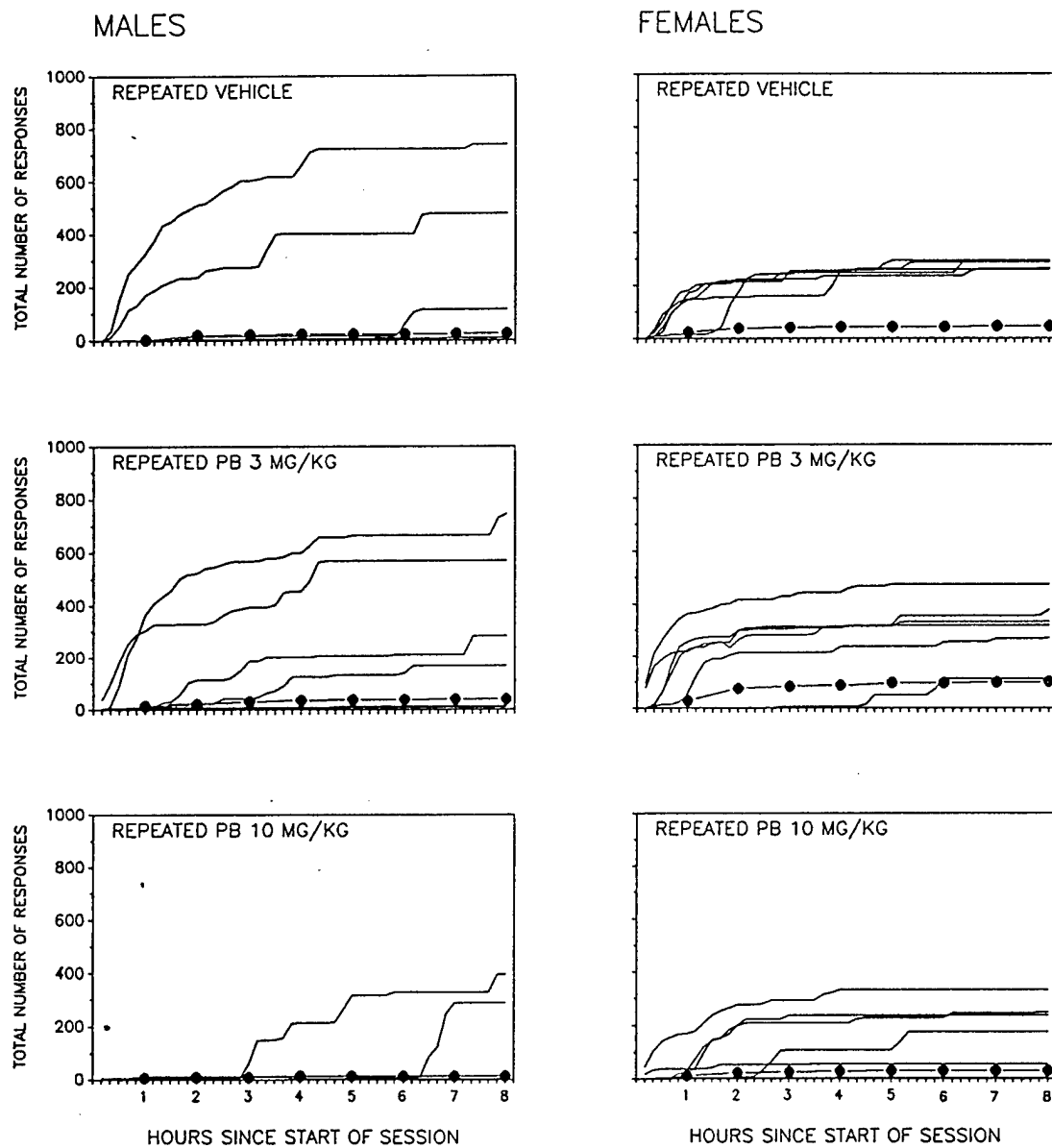


TABLE 1

Average (range) serum and brain cholinesterase levels in male and female rats following acute and repeated pyridostigmine bromide administration

Serum cholinesterase

<u>Acute</u>	Control	PB 3 mg/kg	PB 10 mg/kg
Male	15.50 (3.66-26.59)	14.43 (12.50-17.20)	11.74 (6.20-19.86)
Female	39.40 (29.20-56.00)	22.20 (11.50-20.60)	16.26 (7.06-21.31)

Repeated

Male	18.30 (9.57-27.36)	11.00 (9.57-12.58)	13.10 (8.97-18.39)
Female	33.63 (14.80-44.60)	16.11 (5.52-28.00)	8.71 (0-14.50)

Brain cholinesterase

<u>Acute</u>	Control	PB 3 mg/kg	PB 10 mg/kg
Male	41.87 (34.14-49.59)	41.83 (35.50-53.30)	48.23 (37.40-60.30)
Female	44.06 (30.21-57.90)	49.15 (36.70-61.60)	41.15 (32.70-49.60)

Repeated

Male	42.43 (38.20-50.10)	44.30 (24.60-62.70)	43.06 (33.20-55.20)
Female	38.85 (36.10-41.60)	36.40 (33.70-39.10)	45.85 (35.20-56.50)

Tables I1-I3, Figures I1-I6

Karlix, Freiburger, Hoy, Tebbett, Wielbo, Schmidt, Meyers & van Haaren

Manuscript submitted for Publication

Table I. Effects of Permethrin on lymphocytes stimulated via PHA, PMA, and MLR

<u>Perm ($\mu\text{g/ml}$)</u>	<u>PHA test(cpm)</u>	<u>PMA test(cpm)</u>	<u>MLR test(cpm)</u>
Perm 0	34286 \pm 1455	2237 \pm 143	9779 \pm 1336
Perm 10	8998 \pm 186*	1318 \pm 237*	6979 \pm 1235
Perm 25	4675 \pm 768*	977 \pm 140*	5494 \pm 869*
Perm 50	3927 \pm 206*	2190 \pm 1080	2643 \pm 785*
Perm 100	2141 \pm 220*	634 \pm 47*	704 \pm 206*
Perm 200	2251 \pm 395*	1005 \pm 170*	584 \pm 109*
Perm 300	3293 \pm 461*	338 \pm 57*	1005 \pm 388*

*indicates values that are significantly different from Perm 0 ($P < 0.05$)

Table II. Effects of PB on lymphocytes stimulated via PHA, PMA, and MLR

<u>PB (μg/ml)</u>	<u>PHA test(cpm)</u>	<u>PMA test(cpm)</u>	<u>MLR test(cpm)</u>
PB 0	24465 ±665	28330 ±717	5106 ±512
PB 10	24285 ±858	28299 ±1114	4721 ±659
PB 25	21710 ±673	28661 ±1432	4717 ±789
PB 50	21289 ±3668	28558 ±1123	4518 ±789
PB 100	21803 ±2499	24720 ±2077	3738 ±225
PB 200	21314 ±1056*	23641 ±1843	3143 ±24
PB 300	19539 ±38085	23423 ±1605	2290 ±225

*indicates values that are significantly different from Perm 0 (P<0.05)

Table III. Effects of DEET on lymphocytes stimulated via PHA, PMA, and MLR

<u>DEET(μg/ml)</u>	<u>PHA test(cpm)</u>	<u>PMA test(cpm)</u>	<u>MLR test(cpm)</u>
DEET 0	14720 \pm 1652	33284 \pm 610	4787 \pm 671
DEET 10	16583 \pm 1251	35341 \pm 1486	3871 \pm 628*
DEET 25	11793 \pm 894	30276 \pm 1862	3004 \pm 645*
DEET 50	11252 \pm 907	26634 \pm 630*	2004 \pm 175*
DEET 100	5495 \pm 269*	17707 \pm 1163*	760 \pm 171*
DEET 200	912 \pm 152*	8110 \pm 839*	539 \pm 73*
DEET 300	335 \pm 195*	2144 \pm 222*	271 \pm 114*

*indicates values that are significantly different from Perm 0 ($P < 0.05$)

Fig 1. Effects of Permethrin on lymphocytes stimulated via PHA, PMA, and MLR

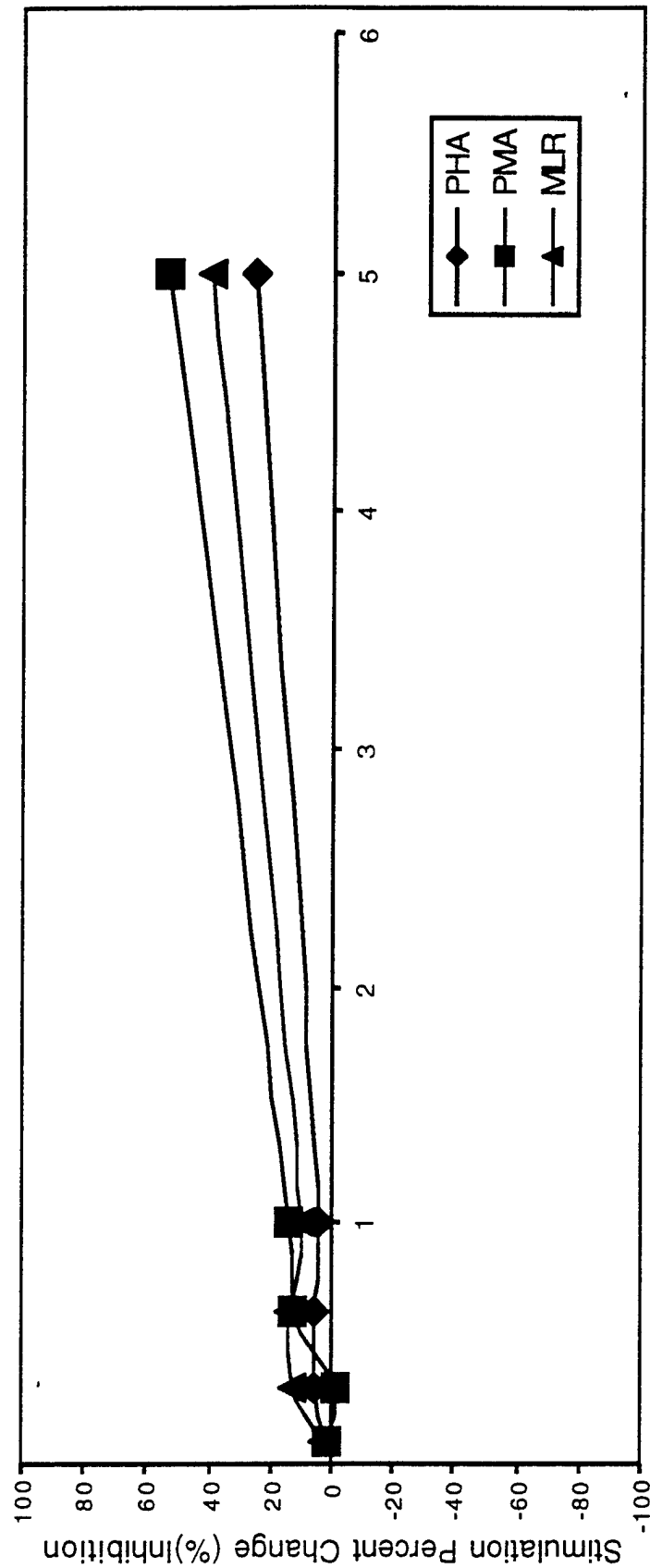


Fig 2. Effects of permethrin on lymphocytes stimulated via PHA, PMA, and MLR

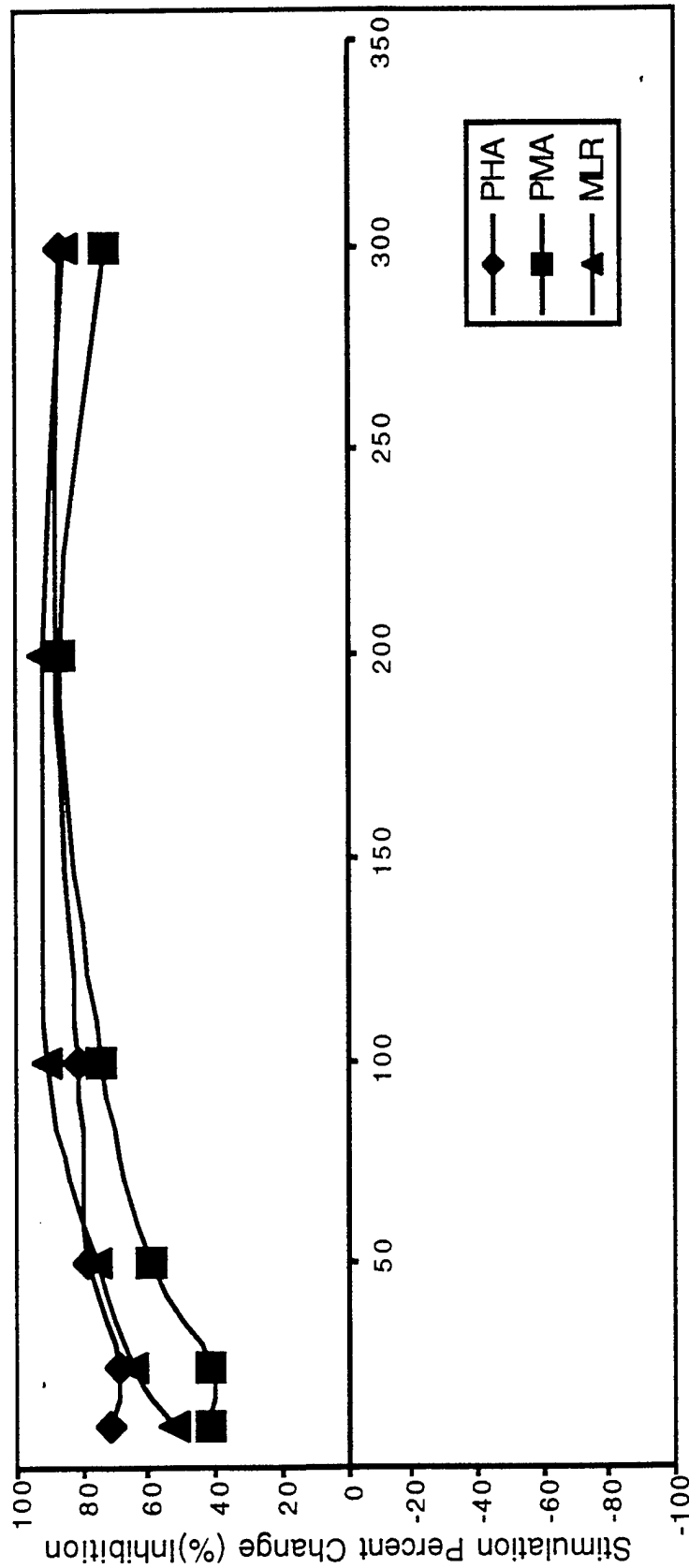
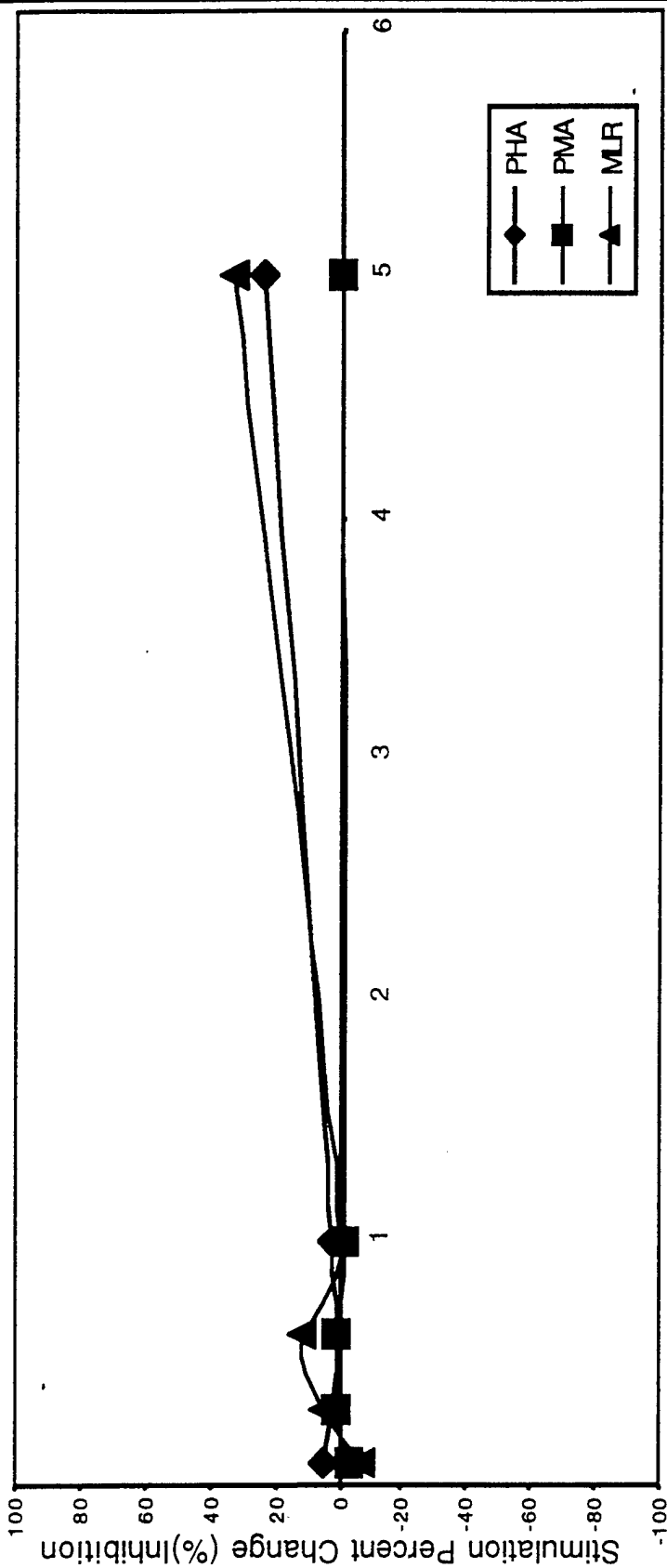
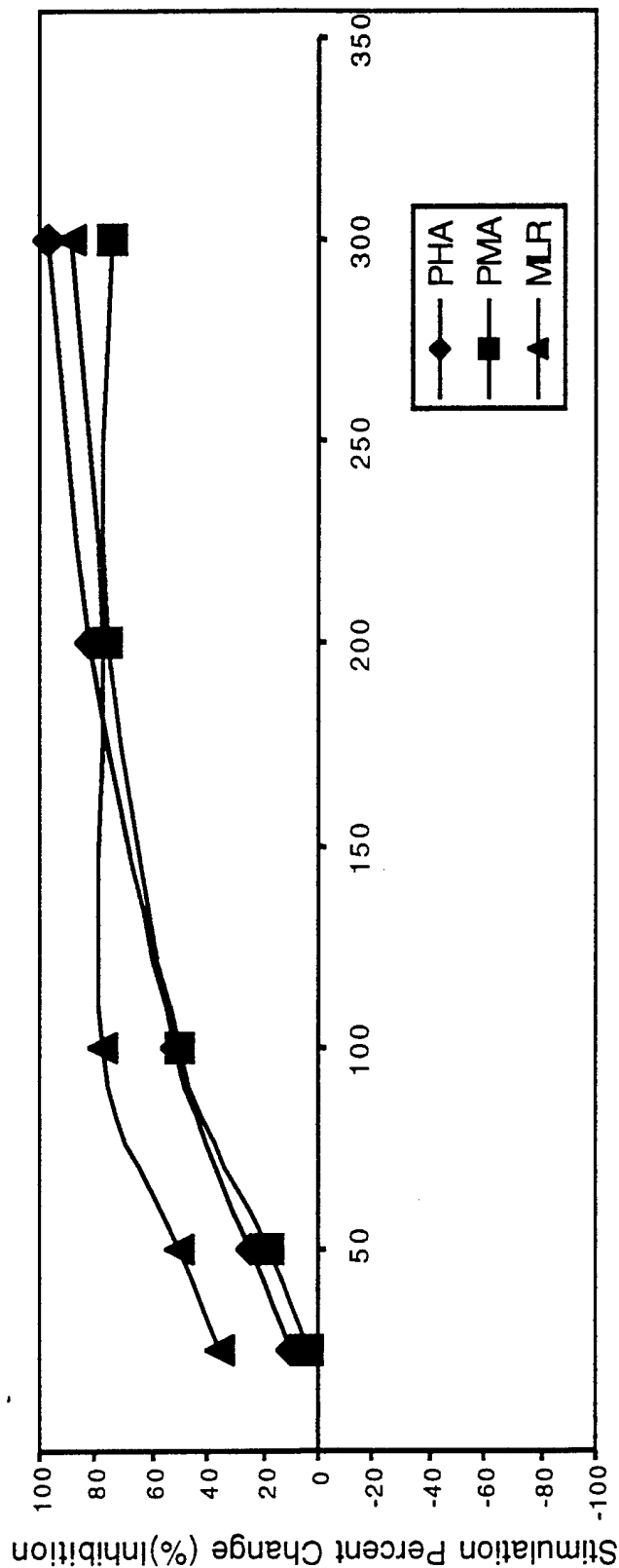


Fig 3. Effects of DEET on lymphocytes stimulated via PHA, PMA, and MLR



DEET concentration 0.1ug/ml - 5ug/ml

Fig 4. Effects of DEET on lymphocytes stimulated via PHA, PMA, and MLR



DEET concentrations 25-300ug/ml

Fig 5. Effects of pyridostigmine bromide on lymphocytes stimulated via PHA, PMA, and MLR

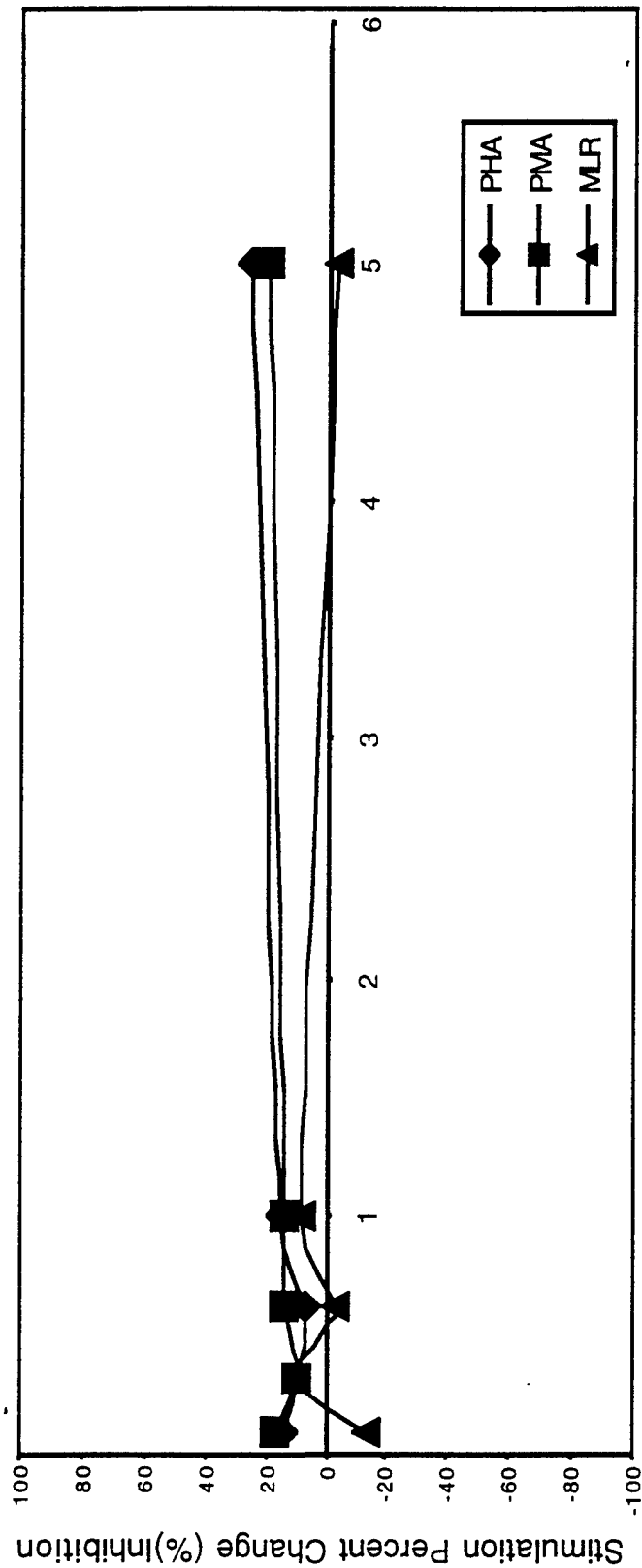


Fig 6. Effects of PB on lymphocytes stimulated via PHA, PMA, and MLR

